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(19) JAPANESE PATENT OFFICE

PATENT ABSTRACTS OF JAPAN

(11) Publication number: 10197481 A

(43) Date of publication of application: 31 . 07 . 98

(51) Int. Cl G01N 27/447
C07K 1/13
C07K 5/103
C07K 7/06

(21) Application number: 09267974

(22) Date of filing: 12 . 09 . 97

(30) Priority: 14 . 11 . 96 JP 08318762

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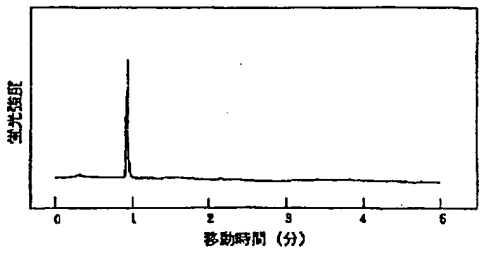
(54) ELECTROPHORETIC MARKER FOR FLUORESCENCE DETECTING ISOELECTRIC POINT

(57) Abstract:

PROBLEM TO BE SOLVED: To provide an electrophoretic marker for fluorescence detecting isoelectric point used for confirming the isoelectric point of a sample material in a fluorescence detecting isoelectric point electrophoresis method.

SOLUTION: A fluorescent material-label oligopeptide for fluorescence detecting isoelectric point electrophoresis having a high isoelectric point pI is obtained by providing a fluorescent group-generating pigment in the SH group of the cysteine of oligopeptide. Since the oligopeptide and the fluorescent group-generating pigment can be bonded to each other under a mild condition, the number and position of a fluorescent material bonded to the oligopeptide are controlled and, accordingly, an obtained electrophoretic marker for fluorescence detecting isoelectric point shows a single sharp pI peak, has a high shift stability, and can be used for fluorescence capillary isoelectric point electrophoresis also.



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LAIMS

:laim(s)]
:laim 1] The marker for fluorescence detection isoelectric focusing characterized by being the marker for fluorescence :tection isoelectric focusing used in fluorescence detection isoelectric focusing, and being the oligopeptide containing at ast one cysteine which combined fluorophore coloring matter with the sulfhydryl group.
:laim 2] The marker for fluorescence detection isoelectric focusing according to claim 1 with which said association is ıaracterized by being one chosen from thioester, dithio ester, and the group that consists of thioether association at least.

:laim 3] It is the amino acid which has the radical in which said oligopeptide emits a proton and has a negative charge at ast. Amino acid and/of nj individual which set to Kj the acid dissociation constant of the radical which emits a proton ıd has a negative charge Or it is the amino acid which has the radical which receives a proton and has positive charge. he amino acid of ni individual which sets to Ki the acid dissociation constant of the radical which receives a proton and ıs positive charge is included. When referred to as Z=sigma i(ni/(1+Ki/[H+]))-sigmaj (nj/(1+ [H+]/Kj)) | When filling |<0.01 - Marker for fluorescence detection isoelectric focusing given in the claim of either [which is characterized by :ing 3<pI<10 when log ([H+]) is set to pI] claim 1 or the either of 2.
:laim 4] Said oligopeptide Gly-Cys-Tyr-Lys-Arg, Gly-Cys-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-yr-Lys-Lys, Gly-Cys-Tyr-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, ly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-sp-Asp-Arg, Gly-Cys-Glu-Glu, The marker for fluorescence detection isoelectric focusing given in one claim of claims -3 characterized by being at least one chosen from the group which consists of Gly-Cys-Asp-Asp-Asp.
:laim 5] Said fluorophore coloring matter is a rhodamine and a fluorescein. Marker for fluorescence detection isoelectric ›cusing given in one claim of claims 1-4 characterized by being fluorophore coloring matter chosen from the group hich consists of cyanine, the India cyanine, the India carbocyanine, the pyronin, RUSHIFA yellow, quinacrine, squaric :id, a coumarin, fluoro ANSE nil maleimide, and an anthracene.
:laim 6] The marker for fluorescence detection isoelectric focusing given in one claim of claims 1-5 to which said uorescence detection isoelectric focusing is characterized by being fluorescence detection capillary tube isoelectric ›cusing.
:laim 7] Gly-Cys-Glu-Tyr-Tyr-Lys-Lys which combined fluorophore coloring matter with the sulfhydryl group of a ysteine, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-is, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu from -- becoming ıarker kit for fluorescence detection isoelectric focusing.
:laim 8] Gly-Cys-Tyr-Lys-Arg which combined fluorophore coloring matter with the sulfhydryl group of a cysteine, ily-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, ily-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-ısp-Asp-Arg, and Gly-Cys-Glu-Glu from -- becoming marker kit for fluorescence detection isoelectric focusing.

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)ETAILED DESCRIPTION

Detailed Description of the Invention]
0001]
Field of the Invention] This invention relates to the marker for fluorescence detection isoelectric focusing and marker kit which are used in fluorescence detection isoelectric focusing.
0002]
Description of the Prior Art] If the particle which has a charge on the surface of a molecule is made to float to an lectrolytic solution and a current is passed The phenomenon which a particle moves toward the direction of each lectrification and objection is observed by Picton, H., and Linder and S.E. in 1892. Since, Since the phenomenon of lectrophoresis comes to be used as a means of the separation analysis of the matter and Tiselius completes equipment in 937, the application side has been quickly expanded to today.
0003] In pH of the value which has each amphoteric electrolyte after developing the ampholine Vesterberg, O., and hose Svensson and H. are synthetic amphoteric electrolytes in 1966 to general electrophoresis using the difference of ıe electrification condition in a certain specific pH, the net charge became zero and isoelectric focusing using the henomenon which stops migrating was established.
0004] The value of pH from which net charge serves as zero here is called isoelectric point of the matter. In isoelectric ocusing, in pH inclination formed in the support for migration, a sample is condensed by the location equal to the soelectric point, and stands it still.
0005] Thus, since a sample is condensed in focus and separated, it is used for separation and analysis of the matter as an lectrophoresis method with very high separability.
0006] Moreover, since the capillary electrophoresis which performs electrophoresis in the capillary tube (capillary tube) ıade in the fused silica the bore of 5-100 micrometers and whose die length are 30-100cm is devised and it has high eparability by the minute amount test sample for chemical analysis in recent years, it has been applied not only to protein ut to separation and analysis of inorganic ion, and a low molecular weight compound and a nucleic acid.
0007] Moreover, the attempt which quantifies concentration is also made by equipping the end of a capillary tube with a etector and detecting the separated sample component.
0008]
Problem(s) to be Solved by the Invention] As the detection approach, it is mainly irradiating ultraviolet radiation or the ight conventionally, and the approach (the ultraviolet visible detecting method) of detecting from change of the quantity f light by those light being absorbed by the sample component is common.
0009] As the still high sensitivity detection approach, the indicator of the test sample for chemical analysis is beforehand arried out with a fluorescent material, light is irradiated at the separated sample component and the detection approach the fluorescence detecting method) which quantifies concentration can be considered by detecting the fluorescence mitted.
0010] For this reason, in carrying out the indicator of the test sample for chemical analysis with a fluorescent material When a test sample for chemical analysis is protein, the technique of combining the proteinic amino group and a proteinic luorescent material chemically as a fluorescent material which is common, for example, is combined with the amino group Fluorescein isothiocyanate () [fluorescein] isothiocyanate, FITC, tetramethylrhodamine isothiocyanate tetramethylrhodamine isothiocyanate, TRITC), etc. are known (the Ono ****, Yuichi Kanaoka, and Fumio Sakiyama -- he Maeda ****) Proteinic chemical modification (below), Japan Scientific Societies Press, 1981.

0011] On the other hand, quantification becomes sufficiently possible about the sample component separated by this luorescence detecting method also in the above-mentioned capillary tube isoelectric focusing, and the possibility as a roteinic super-high sensibility analysis technique is high by that high separability and detection sensitivity.
0012] However, in isoelectric focusing, since the isoelectric point (pI) is presumed from the static position in the inside f the support for migration of the separated sample, it is required to clarify pH inclination formed in the support for nigration, and the standard marker for it is needed.
0013] A this object sake, for example, protein whose pI is clear from the former for example, trypsinogen (pI=9.30) and entile lectin (8.00 pI=7.80, 8.20) -- Homo sapiens hemoglobin C (pI=7.50), Homo sapiens hemoglobin A (pI=7.10), A iorse myoglobin (pI=7.00), Homo sapiens carbo nick anhydrase (pI=6.50), Cow carbo nick anhydrase (pI=6.00), beta actoglobulin B (pI=5.10), Since the fluorescence emitted from these protein itself is very feeble, it is very difficult to use . phycocyanin (pI=4.65), amyloglucosidase (pI=3.50), etc. as the above-mentioned marker as it is.
0014] Then, the approach of carrying out the indicator of the protein with which above pI is clear as one approach with a uitable fluorescent material is considered to be the need.
0015] However, since the number and its dissociation condition of the amino group of N-end in protein and the amino group of a lysine side chain become the big factor which determines proteinic pI (the ******** experiment lecture 2, roteinic chemistry (above), the Japanese Biochemical Society, 1987), if the general indicator approach which carries out hemical bond qualification of the proteinic amino group with a fluorescent material is used, the own isoelectric point of rotein will change a lot.
0016] Furthermore, although the number and location of a fluorescence marker to combine serve as unspecified and it is ne protein as a result since many amino acid in which a fluorescence marker and a reaction are possible exists in protein Since it becomes the mixture in which two or more pI(s) are shown (Flatmark, T., Vesterberg, O., Acta Chem. Scand., 20, 497-1503, 1966), There is a problem that it becomes difficult to make clear to accuracy pH inclination formed into the upport for migration.
0017] Furthermore, proteinic tertiary structure changes with fluorescent material indicators (224 Williamson, A.R., Kreth, H.W., Ann.N.Y.Acad.Sci., 209,211- 1973), and own chemical stability of protein worsens. This poses a problem in espect of the preservation stability as a marker.
0018] Therefore, in fluorescence detection isoelectric focusing, pI cannot use beforehand what carried out the indicator o the clear protein which exists conventionally with the fluorescent material as a marker.
0019] this invention person etc. inquires wholeheartedly in order to solve the above-mentioned trouble, the oligopeptide ontaining a cysteine with fluorophore coloring matter shows pI peculiar to a single, and covers the range of pI value 3<pI<10) big moreover, and he finds out excelling also in preservation stability further, and came to complete this nvention.
0020]
Means for Solving the Problem] In fluorescence detection isoelectric focusing, in order that this invention may make lear to accuracy pH inclination formed into the support for migration, by choosing suitable oligopeptide and carrying out n indicator with a still more suitable fluorescent material, it shows uniform pI and relates to the marker for fluorescence letection isoelectric focusing moreover characterized by preservation stability being high.
0021] That is, this invention is a marker for fluorescence detection isoelectric focusing used in fluorescence detection soelectric focusing, and relates to the marker for fluorescence detection isoelectric focusing characterized by being the oligopeptide containing the cysteine which combined fluorophore coloring matter. The marker for fluorescence detection soelectric focusing characterized by for this invention being a marker for fluorescence detection isoelectric focusing used n fluorescence detection isoelectric focusing, and being the oligopeptide containing at least one cysteine which combined luorophore coloring matter with the sulfhydryl group is offered in more detail.
0022] Moreover, this invention offers the marker for fluorescence detection isoelectric focusing with which said ssociation is characterized by being one chosen from thioester, dithio ester, and the group that consists of thioether association at least.
0023] Furthermore, this invention is amino acid which has the radical in which said oligopeptide emits a proton and has a negative charge at least. Amino acid and/of nj individual which set to Kj the acid dissociation constant of the radical which emits a proton and has a negative charge Or it is the amino acid which has the radical which receives a proton and has positive charge. The amino acid of ni individual which sets to Ki the acid dissociation constant of the radical which receives a proton and has positive charge is included. When referred to as Z=sigma i(ni/(1+Ki/[H+]))-sigmaj (nj/(1+

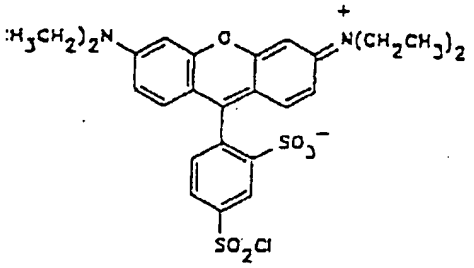
H+]/Kj)) | When filling Z|<0.01 - When log ([H+]) is set to pI, it is offering the marker for fluorescence detection soelectric focusing characterized by being 3<pI<10.
0024] Said oligopeptide this invention Moreover, Gly-Cys-Tyr-Lys-Arg, Gly-Cys-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Tyr-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, Gly-Cys-Glu-Glu or Gly-Cys-Asp-Asp-Asp, and the marker for fluorescence detection isoelectric focusing that crawls and is characterized by being the combination of shoes are offered.
0025] Furthermore, for this invention, said fluorophore coloring matter is a rhodamine and a fluorescein. It is offering the marker for fluorescence detection isoelectric focusing characterized by being fluorophore coloring matter chosen from the group which consists of cyanine, the India cyanine, the India carbocyanine, the pyronin, RUSHIFA yellow, quinacrine, squaric acid, a coumarin, fluoro ANSE nil maleimide, and an anthracene.
0026] Moreover, this invention offers the marker for fluorescence detection isoelectric focusing with which said fluorescence detection isoelectric focusing is characterized by being fluorescence detection capillary tube isoelectric focusing.
0027] Furthermore, this invention combined fluorophore coloring matter with the sulfhydryl group of a cysteine. Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, The marker kit for fluorescence detection isoelectric focusing which consists of Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu is offered.
0028] Gly-Cys-Tyr-Lys-Arg moreover, this invention combined fluorophore coloring matter with the sulfhydryl group of a cysteine -- Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu from -- the becoming marker kit for fluorescence detection isoelectric focusing is offered.
0029] Hereafter, it is based on the gestalt of operation and this invention is explained.
0030]
Embodiment of the Invention]
Oligopeptide) Especially in this invention, oligopeptide is used from various ampholite usable as a marker. In isoelectric focusing, as a sample usually used, the thing of a principal component has much protein etc., therefore it is because it is desirable to use the oligopeptide which consists of same dissociable group in order to check pI value of this sample.
0031] However, when oligopeptide is a thing containing not much many amino acid, in case a radical including fluorophore is combined, existence of two or more reacting points, mixture of the by-product by the reaction condition. etc. may be produced, a sharp peak with the single marker acquired in this case will be given, and it is not desirable.
0032] Therefore, in this invention, what is necessary is just the oligopeptide which has sufficient amino acid radical for there to be especially no limit in the number of the amino acid which forms oligopeptide, and show the target pI.
0033] The oligopeptide which consists of amino acid as an amphoteric compound has various acidic groups, a basic group and an amino terminal amino group, and a C terminal carboxyl group as an acid-base dissociable group. For example, as an amino acid radical which has an acidic group, it is an aspartic acid, glutamic acid, a cysteine, a thyrosin. etc., and they are a lysine, an arginine, a histidine, etc. as an amino acid radical which has a basic group.
0034] Oligopeptide shows a specific number of charges in a certain pH with these acid-base dissociable groups. Therefore, the number of charges as the whole oligopeptide is able to calculate from the class and number of these acid-base dissociable groups.
0035] For example, it becomes possible to presume the number of charges (Z) as the whole oligopeptide by the following formula.
0036] The amino acid of nj individual which is the amino acid which has the radical which emits a proton and has a negative charge here, and sets to Kj the acid dissociation constant of the radical which emits a proton and has a negative charge, The total number of charges (Z) of the oligopeptide containing the amino acid of ni individual which is the amino acid which has the radical which receives a proton and has positive charge, and sets to Ki the acid dissociation constant of the radical which receives a proton and has positive charge It is given by Z=sigma i(ni/(1+Ki/[H+]))-sigmaj (nj/(1+[H+]/Kj)) (486 a chemical field, 36,470- 1982).
0037] Furthermore, in this formula, -log([H+]) =pH in Z= 0 serves as the isoelectric point pI of that oligopeptide.
0038] Therefore, it becomes possible by using this formula to choose an amino acid radical required for the oligopeptide

vhich shows pI used as the object. Moreover, it is required for the variation of a charge to change of pH [in / about the onvergency to the isoelectric point / the isoelectric point of a peptide] and dZ/dpH to be large (1484 1479- Shimura, K., nd Kasai, K., Electrophoresis, 16, 1995). In order for a dZ/dpH value to become large, it is necessary for a dissociable roup with the electric dissociation exponent value near the value of the isoelectric point of the peptide to exist.
0039] Under the present circumstances, it reacts to the halo acetyl derivative of a fluorochrome or a maleimide erivative, and very alternative and high yield, and a labeled compound is generated. As an approach of combining the adical which furthermore contains fluorophore, when combining with the sulfhydryl group of the cysteine of this ligopeptide, basic dissociation by the amino terminal amino group will also be demonstrated, and the convergent good ndicator peptide which has pI before and after pH7.6 which is electric dissociation exponent of the amino terminal amino roup will be obtained.
0040] In order to enable this presumption and to choose a required amino acid radical, as an electric dissociation xponent which the dissociable group of the above-mentioned acid-base nature has For example, alpha-carboxyl (C erminal) (3.6), beta-carboxyl (Asp) (3.95), gamma-carboxyl (Glu) (4.45), an imidazole (His) (6.45), alpha-amino (amino erminal) (7.6), a thiol (Cys) (8.5), phenol nature hydroxy (Tyr) (9.8), epsilon-amino (Lys) (10.2), a GUANIJIUMU adical (Arg) (12.5), etc. are usable.
0041] For example, in order to make pI small, to choose an aspartic acid or glutamic acid and to enlarge pI, the thing vhich choose an arginine or a lysine and for which alpha-amino group is made free is also still more possible. What is ecessary is similarly, just to choose a thyrosin and a histidine, in order to carry out pI among them.
0042] Especially in this invention, it is three or more as range of pI, and 11 or less thing is desirable. It is three or more urther especially especially as range of pI, and ten or less thing is desirable.
0043] Furthermore, in this invention, it is not restricted especially about the synthetic approach of the oligopeptide ontaining the amino acid chosen desirably. The composition (the foundation of peptide synthesis, such as a liquid phase rocess and a solid phase technique, an experiment, Nobuo Izumiya, Tetsuo Kato, the Aoyagi ****, the near Michinori vork, Maruzen 1985) by the general synthetic approach or composition by the automatic composition approach is referably usable.
0044] Moreover, in this invention, a required amino acid radical exists, and if a labile point is still more controllable, the ll or a part is suitably usable [the oligopeptide containing at least one cysteine of the natural product origin]. pecifically, oxytocin, vasopressin, the oligopeptide of the vasotocin origin, and the oligopeptide of the somatostatin rigin are mentioned.
0045] It is also possible to compound the oligopeptide which combines an amino acid radical still more nearly required or the oligopeptide of the natural product origin by chemosynthesis, and has a desirable amino acid radical. Under the resent circumstances, there is especially no limit in the number of cysteines, or the array location of a cysteine.
0046] (Fluorophore) What is necessary is not to be restricted especially about usable fluorophore's class, but just to nclude the fluorescence coloring matter generally known in this invention.
0047] For example, a rhodamine, a fluorescein What is necessary is just cyanine, the India cyanine, the India arbocyanine, the pyronin, RUSHIFA yellow, quinacrine, squaric acid, a coumarin, fluoro ANSE nil maleimide, etc. In his invention, a rhodamine, cyanine dye, etc. are especially desirable and it is usable.
0048] It is desirable to introduce rhodamine fluorophore (Handbook of Fluorescent Probes and Research Chemicals, 5th dition MOLECULAR PROBES, INC., 1992) especially in this invention.
0049] This rhodamine fluorophore has the fluorescence which has absorption maximum (molar extinction coefficient 3,000) in 556nm in a methanol, and makes 576nm the maximum.
0050] Furthermore in this invention, there is an aromatic heterocyclic compound or polycyclic aromatic hydrocarbon as sable fluorophore suitably (for example, fluorescence phosphorimetry, Taiji Nishikawa, Keizo Hiraki work, KYORITSU SHUPPAN, 1989 reference). For example, those derivatives, such as an anthracene, naphthalene, a henanthrene, a quinoline, a pyrene, and perylene, are especially suitably usable. It becomes such hydrocarbon luorophores combinable with oligopeptide suitably by preparing a suitable joint radical.
0051] (Joint radical) In this invention, there is especially no limit in the class of joint radical which combines a luorophore radical and the sulfhydryl group of the cysteine of oligopeptide. The radical which contains fluorophore hrough a joint radical may combine with said sulfhydryl group, and may join together through a suitable joint radical.
0052] In this case, it is desirable that a radical including fluorophore combines with said sulfhydryl group by for example, thioester association, the dithio ester bond, thioether association (sulfide association), etc. Especially in this

ıvention, thioether association is desirable.
)053] The introductory approach of a (fluorophore dye base)-(joint radical)-S-cysteine joint radical does not have specially a limit. When the radical containing fluorophore coloring matter has a halogenation methyl group (for xample, an iodine methyl group, -CH2I), an activity ester group, an acid chloride, an acid anhydride, a maleimide ıdical, etc., these active groups and reactions are possible for it, and 2 functionality radical in which the sulfhydryl group f a cysteine and a reaction are still more possible is desirable.
)054] For example, when a rhodamine fluorophore radical has an acid-chloride radical (for example, coloring matter-O2Cl) Thioamide association (coloring matter-SO2NH(CH2)2NH2) is first formed by the reaction with diamine (for xample, ethylenediamine). Making it react with monoiodoacetic acid N-malic acid imide ester furthermore, coloring ıatter-SO2NH(CH2)2NHCOCH2I obtained reacts with the sulfhydryl group of the cysteine of oligopeptide selectively asily, and forms thioether association.
)055] Coloring matter - An SO2NH(CH2)2NHCOCH2-S-cysteine book reaction can be carried out on mild conditions, nd does not give change to other oligopeptide parts and fluorochrome sections.
)056] Furthermore, since a reaction is made possible only at said sulfhydryl group when two or more labile points are in ligopeptide, selection of a reaction condition, a reaction reagent, etc. is possible. Since this selection is enabled, the :chnique of general organic synthesis is suitably usable.
)057] For example, if required, it is one of the desirable technique to protect beforehand other reactant substituents (for xample, the amino group, a phenolic group, etc.).
)058] In this invention, the purity and preservation stability of the acquired marker for fluorescence detection capillary ıbe isoelectric focusing can be checked with isoelectric focusing.
0059] If still more nearly required, it can refine simple using high performance chromatography. Efficient separation ·urification is attained by using an opposite phase system column, especially the bulking agent to which the chemical ·ond of the octadecyl radical was carried out in that case. It is usable wavelength suitable [280nm] for detection.
0060] (pI measurement) In this invention, it is measurable pI of the marker for fluorescence isoelectric focusing ·erforming isoelectric focusing, and measuring mobility with the commercial marker for isoelectric focusing, or by neasuring directly pH of the migration location of an indicator peptide.
0061] Slab (plane) gel electrofocusing which uses polyacrylamide as a base material is desirable, and isoelectric focusing ; usable (Righetti, P.G., Isoelectric Focusing:Theory, Methodology and Applications, Elsevier, Amsterdam, 1983 eference).
0062] The detection by the dyeing approach is suitably usable in a general approach (for example, Coomassie Brilliant 3lue-R250 dyeing).
0063] Furthermore, the detection by fluorometry is suitably usable in a general approach, for example, the approach by he densitometer, (for example, the Shimadzu make, Shimazu two-wave premature start spot scanning densitometer :S9300PC).
0064] (Capillary tube isoelectric focusing) There is especially no limit in the equipment for capillary tube isoelectric ocusing which can use the indicator oligopeptide concerning this invention as a marker.
0065] Generally, with capillary tube isoelectric-focusing equipment, the peptide which carried out the indicator of the luorescent material is condensed by the location of pH formed in the support for migration according to each pI, and tands it still. Furthermore, these locations may be checked by detecting the fluorescence generated by excitation light :xposure.
0066] In this invention, although especially the excitation light source is not restricted, especially its activity of the laser vhich enables the stable optical exposure is desirable.
0067] For example, in this invention, helium neon laser is suitably usable as an excitation light to the rhodamine coloring natter which is fluorophore.
0068] The gradient of pH formed in the support for migration by the fluorescence detector formed in the end of a :apillary tube can be checked.
0069] As explained in the top, the marker for fluorescence detection isoelectric focusing of this invention is compounded ›y combining the radical which has the fluorophore of oligopeptide, and has stability uniform [pI] and high moreover. Therefore, in fluorescence detection isoelectric point migration, it becomes a marker for the high sensitivity analysis at he time of checking the isoelectric point of the sample matter which migrated based on the gradient of pH formed in the ;upport for migration.

0070] Therefore, the marker for fluorescence detection isoelectric focusing of this invention becomes usable also to the apillary tube isoelectric point migration approach.
0071] Hereafter, although this invention is explained still more concretely based on an example, this invention is not imited to the following examples, unless the summary is exceeded.
0072] (Marker kit for fluorescence detection isoelectric focusing) As explanation was given [above-mentioned], oligopeptide usable to the marker for fluorescence isoelectric focusing concerning this invention combines [choose imely and] and is usable.
0073] That is, the marker concerning this invention shows the good focus nature which is what shows good coincidence of rear-spring-supporter calculated value and an actual measurement as pI to the range of 3-10 (specifically mentioned to table 1). Furthermore, the marker concerning this invention is the oligopeptide of low molecular weight, and even if it loubles two or more sets of them and uses it under various isoelectric-focusing conditions, the focusing of it is ndependently carried out to each pI value, respectively. Based on the above-mentioned table 1, the suitable thing for vhich number selection is made becomes possible about the marker concerning this invention which is in within the imits to specifically use as a marker as the above-mentioned combination.
0074] for example, the number shown in a table 1 -- ten kinds of combination (Gly-Cys-Glu-Tyr-Tyr-Lys-Lys) of 6, 7, 8, ', 10, 11, 12, 13, 14, and 15 Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu re one example of a desirable kit. These cover pI of the large range and the pI also serves as abbreviation regular ntervals.
0075] moreover -- the same -- 12 kinds of combination (Gly-Cys-Tyr-Lys-Arg) of 1, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 5 Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu are also one example of a desirable kit.
0076]
Example]
Composition of the marker for fluorescence detection isoelectric focusing)
1) Gly-Cys-Tyr-Lys-Arg, Gly-Cys-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Tyr-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, Gly-Cys-Glu-Glu, preparation of Gly-Cys-Asp-Asp-Asp.
0077] a peptide -- the peptide automatic synthesizer unit made from Applied Biosystems -- Boc -- it compounded based on law (see the new chemistry experiment lecture 1, protein VI, the edited by Japanese Biochemical Society, and 1992), and the high-speed liquid chromatograph refined the obtained oligopeptide. The conditions used for the high-speed liquid chromatograph are column [by YMC CO., LTD.] S-5,120A. The rate of flow used a part for /, and 20ml detection wavelength for 220nm and a mobile phase on the following gradient / 80-minute conditions with -0.1% trifluoroacetic acid of acetonitrile inclination using ODS (30mm bore x250mm overall length).
0078]
Oligopeptide HPLC mobile phase inclination conditions ---------- Gly-Cys-Tyr-Lys-Arg 3%- 23%Gly-Cys-Tyr-Lys-Lys 4.5% - 24.5%Gly-Cys-Tyr-Tyr-Lys-Lys 2.5% - 22.5%Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys 1%- 19%Gly-Cys-Tyr-Lys 3.7% - 23.7%Gly-Cys-Glu-Tyr-Tyr-Lys-Lys 2.3% - 22.3%Gly-Cys-Glu-His-His-His-Arg 1%- 18%Gly-Cys-Glu-His-Arg 0% - 5%Gly-Cys-Glu-His-His 0.1%- 10%Gly-Cys-Glu-Arg 1%- 10%Gly-Cys-Glu-His0%- 5%Gly-Cys-Asp-Asp-His-His 1%- 15%Gly-Cys-Glu-Glu-His 1%- 13%Gly-Cys-Asp-Asp-Arg 1% - 18.5% Gly-Cys-Glu-Glu 1%- 19%Gly-Cys-Asp-Asp-Asp 0.5%- 10% ----------
0079] (2) Preparation of lisamine rhodamine B (LRB) iodoacetamide (Alexander, H., Lewis, A.S., and Ram, R.C., J.Medicinal Chem., 1976, Vol19, 1279-1283)
(a) The 10mg lisamine rhodamine B sulfonyl chloride (structure is shown made in [molecular PUROBUSU] the U.S. and in the following) dissolved in 0.3ml dimethylformamide at mixture (preparation ethylenediamine 104mg of a LRB amine and dimethylformamide 0.4ml) was dropped, and it was made to react at a dark place room temperature for 1 hour.

0080]
Formula 1]

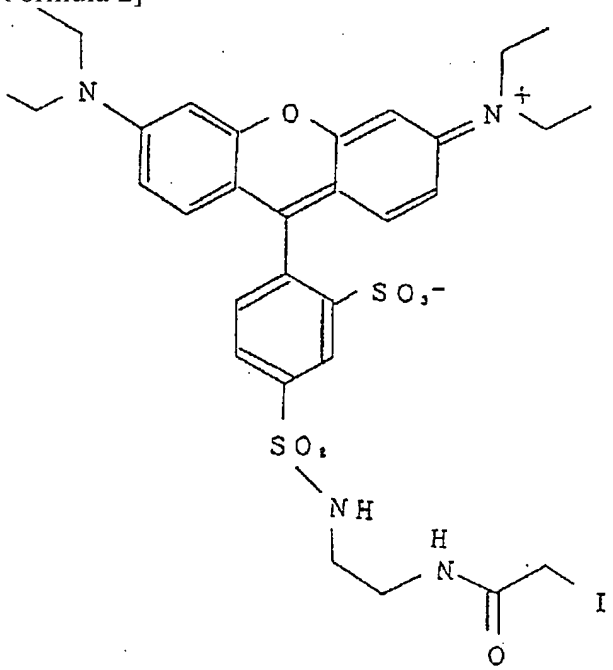


)081] After carrying out evaporation to dryness of the reaction mixture under reduced pressure, it dissolved in 2ml of ɔrmic-acid solutions 20%·acetonitrile-1%, and the whole-quantity style was carried out to Dowex-1 (H1 mold) 1ml olumn which equilibrated with the formic-acid solution 20% acetonitrile-1%, and bypassing fractions were collected, vaporation to dryness was carried out under reduced pressure, and it considered as the crude material of a LRB amine. pectrometry determined the concentration, having dissolved this in acetonitrile 0.4ml and 1.6ml (pH7.5) of 0.1M sodium hosphate buffer solutions, and having used the molar extinction coefficient in 570nm as 93,000. It applied to the pposite phase chromatograph column (TOSOH ODS-80Ts, the diameter of 4.6mm, die length of 25cm) which quilibrated LRB amine crude-material 150nmol with the trifluoroacetic acid solution 25% acetonitrile-0.1%, and it was luted over 30 minutes, having applied the concentration straight-line inclination of 25-55% of acetonitrile, and detected y acting as the monitor of the 280nm absorption. The biggest peak was isolated preparatively (127nmol) and it used for ıe following actuation as a purification LRB amine.

)082] (b) it hardened by drying under reduced pressure of the fraction prepared by iodine acetylation (a) of a LRB mine, and it dissolved in 88micro of 1:4 mixed liquor l of an acetonitrile and the 0.1M sodium phosphate buffer solution ɔH7.5) after that, 12micro of dimethylformamide solutions l of 10mM monoiodoacetic acid N-malic acid imide ester /as added to this, and it was made to react at a room temperature for 1 hour It applied to the opposite phase hromatograph on the same conditions which explained the whole quantity of reaction mixture by (a), the eluted main eak was isolated preparatively as LRB-iodoacetamide (70nmol), and it used for the indicator of a peptide. The structure f LRB-iodoacetamide was shown below.

)083]

Formula 2]

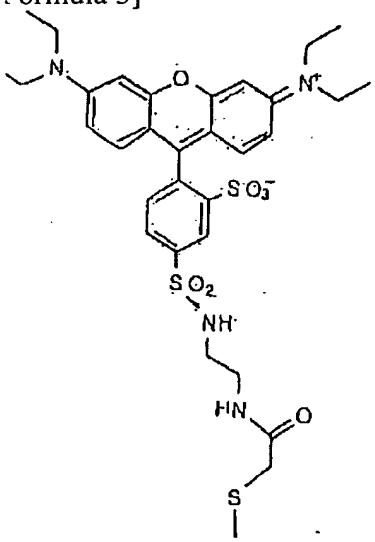


0084] (3) The column solution containing purification coloring matter (LRB-iodoacetamide) 25nmol prepared with the ndicator (2) of a peptide was hardened by drying in the test tube, and it dissolved in acetonitrile 20microl after that.

0micro (concentration 5mM, 50nmol) of peptide water solutions l and the buffer solution (0.1 M Na-Pi, 5mM EDTA, H7.5) which were prepared by (1) were added to this, and it was made to react to it at a room temperature in a dark place vernight. reaction mixture was applied to the opposite phase chromatograph column (TOSOH ODS-80Ts, the diameter f 4.6mm, die length of 25cm) which equilibrated with the trifluoroacetic acid solution 25% acetonitrile-0.1%, and the ıdicator peptide was eluted in the 1ml rate of flow for /, having applied the concentration straight-line inclination of 25-5% of acetonitrile over 30 minutes -- it isolated preparatively. Detection was performed by 280nm absorption. The yield f an indicator peptide was 80%. The concentration of an indicator peptide set the molar extinction coefficient in 570nm › 93,000, and determined it by spectrometry. The structure of oligopeptide Gly-Cys-Glu-Tyr-Tyr-Lys-Lys by which the ıdicator was carried out to below is shown.

)085]

Formula 3]



H-Gly-Cys-Glu-Tyr-Tyr-Lys-Lys-OH

0086] Gly-Cys-Tyr-Lys, (pI of a fluorescent material indicator peptide) Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-Iis-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-Iis-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, Gly-Cys-Glu-Glu, and the above-mentioned fluorescent material ıdicator oligopeptide of Gly-Cys-Asp-Asp-Asp Ampholine PAG Plate (pH 3.5-9.5, Pharmacia Biotech K.K. make) (5% ` --) Isoelectric focusing was performed using 3%C, 24.5mmx110mm magnitude, and 1mm thickness, and mobility with ıe commercial marker for isoelectric focusing (pI calibration kit 3-10, Pharmacia Biotech K.K. make) was measured.

0087] After migration conditions started electrophoresis on the electrical potential difference of 300V, they increased the lectrical potential difference to 1,500V after 30 minutes, and performed migration for 50 minutes.

0088] A photograph was taken after migration termination by irradiating ultraviolet rays through an orange filter about luorescent material indicator oligopeptide, and the migration location was checked.

0089] Then, in order to know the migration location of the commercial marker for isoelectric focusing, page blue 83 tain solution (CBB-R250, the first chemicals company make) dyed gel.

0090] The migration location was checked by removing the background in the water solution of a methanol and an acetic .cid after dyeing.

0091] Moreover, about the above-mentioned fluorescent material indicator oligopeptide of Gly-Cys-Tyr-Lys-Arg, Gly-:ys-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Lys-Lys, and Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, pI was measured by the following .pproaches, and ****** was determined.

0092] That is, the 1x50x115mm polyacrylamide gel (4.2%T, 4.8%C) included by 1/16 dilution of an undiluted solution ıs a carrier ampholite for pH inclination formation on a 1x65x125mm synthetic silica plate by making Pharmalyte 8-10.5 ›y Pharmacia Biotech K.K. into final concentration was produced. the silica plate with which gel rode -- constant emperature -- in order to place on the level cooling plate made to circulate through water and to avoid the effect of the :arbon dioxide gas in air, electrophoresis was performed all over the glove compartment which filled nitrogen gas.

focusing was carried out on the electrical potential difference of about 100 V/cm until the sample which placed the lectrode along with the short piece, added each about 0.5 peptide nmol on the gel front face with a location of 1cm from ıe cathode and anode plate side as a sample, and was added eventually carried out focusing to one place (about 2 hours). ıfter focusing was completed, the temperature of the water through which it circulates to a gel cooling plate was adjusted o that the temperature of gel might become 25 degrees C in the condition [having applied the electrical potential ifference], and pH on the front face of gel was measured at 1cm spacing in accordance with pH inclination. The ŒTOXYpH meter (HM-17MX mold) by Toa Electronics, Ltd. was used for the temperature of gel, and measurement of ·H. pH was plotted to the location of gel, pH inclination in gel was searched for, and the isoelectric point of each luorescent-labeling oligopeptide was determined from the focusing location of fluorescent material indicator ligopeptide.

0093] The migration image of the marker for isoelectric focusing of the obtained fluorescent material indicator ·ligopeptide and marketing was shown in drawing 1 .

0094] What compared the calculated value based on pI and the formula (1) of each fluorophore indicator oligopeptide vhich were obtained was summarized in a table 1.

0095]

A table 1)

·I actual measurement and calculated value = ========== number Structure

Calculated value Actual measurement ---------- 1 Gly-Cys-Tyr-Lys-Arg 10.02 9.002 Gly-Cys-Tyr-.ys-Lys 9.76 8.983 Gly-Cys-Tyr-Tyr-Lys-Lys 9.52 8.914 Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys 9.36 8.855 Gly-Cys-Tyr-Lys :.62 8.306 Gly-Cys-Glu-Tyr-Tyr-Lys-Lys 8.46 8.367 Gly-Cys-Glu-His-His-His-Arg 7.34 7.678 Gly-Cys-Glu-His-Arg '.04 7.249 Gly-Cys-Glu-His-His 6.42 6.7110 Gly-Cys-Glu-Arg 6.066.0411 Gly-Cys-Glu-His 5.48 5.6012 Gly-Cys-Asp-\sp-His-His 5.24 5.3413 Gly-Cys-Glu-Glu-His 4.54 4.3514 Gly-Cys-Asp-Asp-Arg 4.16 4.1015 Gly-Cys-Glu-Glu3.82 :.5916 Gly-Cys-Asp-Asp-Asp 3.38 <3.50(*) = Since the migration location of

==========(*) fluorescent-labeling oligopeptide had crossed the range of the mobility ıf the used commercial marker for isoelectric focusing (pI 9.30-3.50), an actual measurement was not able to be obtained.

0096] (Fluorescence detection capillary tube isoelectric focusing) Fluorescence detection capillary tube isoelectric ocusing performed separation and detection for the obtained fluorescent material indicator oligopeptide (Hjerten, S., ourn al of Chromatography, 347, 191-198, 1985). The dissolution silica capillary tube (GL Sciences make) with the bore ıf 0.05mm which performed coating processing for the inner surface by polyacrylamide, an outer diameter [of).375mm], and an overall length of 22cm was filled with Dilution Pharmalyte (pH 3-10, Pharmacia Biotech K.K. make) :5 times, and it introduced by pouring the 25 time dilution Pharmalyte (pH 3-10) solution containing the oligopeptide Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, 1x10-8M) obtained from the anode plate the account of a top for 1 minute with the ıydrostatic pressure of 5cm of fall.

0097] 20mM phosphoric acid was used as an anolyte, NaOH of 20mM(s) was used as a catholyte, the two-electrodes tub vas made into the same height, and focusing was carried out by 500 V/cm for 5 minutes. Adding the electric field of 500 //cm, by making an anode plate tub high 5cm to a cathode tub, it was moved to the cathode side and the marker which :arried out focusing performed fluorescence detection by laser excitation from the cathode edge in the location of 2cm.

0098] Excitation of a fluorescent material is helium neon laser (the wavelength of 543.5nm, and the output of 1mW). Model; It carries out using 05-LGR-151-S and MERESU griot company make. The band pass filter after condensing the generated fluorescence with a 40 times as many objective lens as this (590nm) It measured with the photo-multiplier model; R1387, Hamamatsu Photonics make) through 30nm bandwidth, model;DIF-BP -3, and the Optical Coatings apan make, and the measurement result was analyzed using the integrator (model;CR4A, Shimadzu Corp. make).

0099] The obtained result was shown in drawing 2 .

0100] According to the above result, fluorescent material indicator oligopeptide is obtained by including fluorophore :oloring matter in oligopeptide by this invention. Under the present circumstances, the oligopeptide containing the luorophore coloring matter which oligopeptide and fluorophore can also pinpoint the number and location of a luorescent material which are further combined with oligopeptide, without doing a failure to oligopeptide and a luorescent material since it may be combined under mild conditions, and is obtained will have extremely stable uniform ;ingle pI. Therefore, the marker for fluorescence detection capillary tube isoelectric focusing acquired in this invention ıecomes usable as a marker at the time of presuming the isoelectric point of the sample matter.

0101]
Effect of the Invention] The marker for fluorescence detection isoelectric focusing concerning this invention used in luorescence detection isoelectric focusing is characterized by being the oligopeptide containing at least one cysteine /hich combined fluorophore coloring matter with the sulfhydryl group. Therefore, the marker concerning obtained this ıvention becomes what was excellent in the chemical stability as compared with the marker based on the usual ıacromolecule protein. Since it is a thing containing at least one cysteine which furthermore combined fluorophore oloring matter with the sulfhydryl group, the marker in which high pI is shown is acquired. pI which the marker acquired hows from it being possible to perform the above-mentioned ligation reaction selectively serves as the shape of a niform and sharp peak.
0102] Therefore, in fluorescence detection isoelectric point migration, it becomes a marker for the high sensitivity nalysis at the time of checking the isoelectric point of the sample matter which migrated based on the gradient of pH ormed in the support for migration, and becomes usable also at the capillary tube isoelectric point migration approach.
0103]
Layout Table]
rray number: -- die-length [of one array]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Tyr Lys Arg

5

ike array]: -- a peptide

rray number: -- die-length [of two arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Tyr Lys Lys

5

ike array]: -- a peptide

rray number: -- die-length [of three arrays]: -- mold [of six arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Tyr Tyr Lys Lys

5

ike array]: -- a peptide

rray number: -- die-length [of four arrays]: -- mold [of seven arrays]: -- amino acid topology: -- class [of straight

配列

Gly Cys Tyr Tyr Tyr Lys Lys

5

hain-like array]: -- a peptide

rray number: -- die-length [of five arrays]: -- mold [of four arrays]: -- amino acid topology: -- class [of straight chain-ike array]: -- peptide array Gly Cys Tyr Lys array number: -- die-length [of six arrays]: -- mold [of seven arrays]: --

配列

Gly Cys Glu Tyr Tyr Lys Lys

5

mino acid topology: -- class [of straight chain-like array]: -- a peptide

rray number: -- die-length [of seven arrays]: -- mold [of seven arrays]: -- amino acid topology: -- class [of straight

配列

Gly Cys Glu His His His Arg

5

hain-like array]: -- a peptide

rray number: -- die-length [of eight arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight

配列

Gly Cys Glu His Arg

5

hain-like array]: -- a peptide

rray number: -- die-length [of nine arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Glu His His

5

ike array]: -- a peptide

rray number: -- die-length [of ten arrays]: -- mold [of four arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide array Gly Cys Glu Arg array number: -- die-length [of 11 arrays]: -- mold [of four arrays]: -- mino-acid topology: -- class [of straight chain-like array]: -- peptide array Gly Cys Glu His array number: -- die-length of 12 arrays]: -- mold [of six arrays]: -- amino-acid topology: -- class [of straight chain-like array]: -- a peptide

配列

ly Cys Asp Asp His His

5

rray number: -- die-length [of 13 arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Glu Glu His

5

ike array]: -- a peptide

rray number: -- die-length [of 14 arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Asp Asp Arg

5

ike array]: -- a peptide

rray number: -- die-length [of 15 arrays]: -- mold [of four arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide array Gly-Cys-Glu-Glu array number: -- die-length [of 16 arrays]: -- mold [of five arrays]: --

配列

Gly Cys Asp Asp Asp

5

mino acid topology: -- class [of straight chain-like array]: -- a peptide

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NOTICES *

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.**** shows the word which can not be translated.
.In the drawings, any words are not translated.

ECHNICAL FIELD

Field of the Invention] This invention relates to the marker for fluorescence detection isoelectric focusing and marker kit hich are used in fluorescence detection isoelectric focusing.

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PRIOR ART

Description of the Prior Art] If the particle which has a charge on the surface of a molecule is made to float to an electrolytic solution and a current is passed, Since the phenomenon which a particle moves toward a direction opposite to each electric charge was observed by Picton, H., and Linder and S.E. in 1892, the phenomenon of electrophoresis comes to be used as a means of the separation analysis of the matter and Tiselius completes equipment in 1937, the application side has been quickly expanded to today.

0003] In pH of the value which has each amphoteric electrolyte after developing the ampholine Vesterberg, O., and whose Svensson and H. are synthetic amphoteric electrolytes in 1966 to general electrophoresis using the difference of the electric charge condition in a certain specific pH, the net charge became zero and isoelectric focusing using the phenomenon which stops migrating was established.

0004] The value of pH from which net charge serves as zero here is called isoelectric point of the matter. In isoelectric focusing, in pH inclination formed in the support for migration, a sample is condensed by the location equal to the isoelectric point, and stands it still.

0005] Thus, since a sample is condensed in focus and separated, it is used for separation and analysis of the matter as an electrophoresis method with very high separability.

0006] Moreover, since the capillary electrophoresis which performs electrophoresis in the capillary tube (capillary tube) made in the fused silica the bore of 5-100 micrometers and whose die length are 30-100cm is devised and it has high separability by the minute amount test sample for chemical analysis in recent years, it has been applied not only to protein but to separation and analysis of inorganic ion, and a low molecular weight compound and a nucleic acid.

0007] Moreover, the attempt which quantifies concentration is also made by equipping the end of a capillary tube with a detector and detecting the separated sample component.

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EFFECT OF THE INVENTION

Effect of the Invention] The marker for fluorescence detection isoelectric focusing concerning this invention used in luorescence detection isoelectric focusing is characterized by being the oligopeptide containing at least one cysteine vhich combined fluorophore coloring matter with the sulfhydryl group. Therefore, the marker concerning obtained this nvention becomes what was excellent in the chemical stability as compared with the marker based on the usual nacromolecule protein. Since it is a thing containing at least one cysteine which furthermore combined fluorophore oloring matter with the sulfhydryl group, the marker in which high pI is shown is acquired. pI which the marker acquired hows from it being possible to perform the above-mentioned ligation reaction alternatively serves as the shape of a niform and sharp peak.
0102] Therefore, in fluorescence detection isoelectric point migration, it becomes a marker for the high sensitivity nalysis at the time of checking the isoelectric point of the sample matter which migrated based on the gradient of pH ormed in the support for migration, and becomes usable also at the capillary tube isoelectric point migration approach.
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配列

Gly Cys Tyr Lys Arg
5

ike array]: -- a peptide

rray number: -- die-length [of two arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Tyr Lys Lys
5

ike array]: -- a peptide

rray number: -- die-length [of three arrays]: -- mold [of six arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Tyr Tyr Lys Lys
5

ike array]: -- a peptide

rray number: -- die-length [of four arrays]: -- mold [of seven arrays]: -- amino acid topology: -- class [of straight

配列

Gly Cys Tyr Tyr Tyr Lys Lys
5

hain-like array]: -- a peptide

rray number: -- die-length [of five arrays]: -- mold [of four arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide array Gly Cys Tyr Lys array number: -- die-length [of six arrays]: -- mold [of seven arrays]: --

配列

Gly Cys Glu Tyr Tyr Lys Lys

5

mino acid topology: -- class [of straight chain-like array]: -- a peptide

rray number: -- die-length [of seven arrays]: -- mold [of seven arrays]: -- amino acid topology: -- class [of straight

配列

Gly Cys Glu His His His Arg

5

hain-like array]: -- a peptide

rray number: -- die-length [of eight arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight

配列

Gly Cys Glu His Arg

5

hain-like array]: -- a peptide

rray number: -- die-length [of nine arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Glu His His

5

ke array]: -- a peptide

rray number: -- die-length [of ten arrays]: -- mold [of four arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide array Gly Cys Glu Arg array number: -- die-length [of 11 arrays]: -- mold [of four arrays]: -- mino-acid topology: -- class [of straight chain-like array]: -- peptide array Gly Cys Glu His array number: -- die-length of 12 arrays]: -- mold [of six arrays]: -- amino-acid topology: -- class [of straight chain-like array]: -- a peptide

配列

ly Cys Asp Asp His His

5

rray number: -- die-length [of 13 arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Glu Glu His

5

ke array]: -- a peptide

rray number: -- die-length [of 14 arrays]: -- mold [of five arrays]: -- amino acid topology: -- class [of straight chain-

配列

Gly Cys Asp Asp Arg

5

ke array]: -- a peptide

rray number: -- die-length [of 15 arrays]: -- mold [of four arrays]: -- amino acid topology: -- class [of straight chain-like array]: -- peptide array Gly-Cys-Glu-Glu array number: -- die-length [of 16 arrays]: -- mold [of five arrays]: --

配列

Gly Cys Asp Asp Asp

5

mino acid topology: -- class [of straight chain-like array]: -- a peptide

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Translation done.]

NOTICES *

ECHNICAL PROBLEM

Problem(s) to be Solved by the Invention] As the detection approach, it is mainly irradiating ultraviolet radiation or the ght conventionally, and the approach (the ultraviolet visible detecting method) of detecting from change of the quantity f light by those light being absorbed by the sample component is common.

0009] As the still high sensitivity detection approach, the indicator of the test sample for chemical analysis is beforehand arried out with a fluorescent material, light is irradiated at the separated sample component and the detection approach he fluorescence detecting method) which quantifies concentration can be considered by detecting the fluorescence mitted.

0010] For this reason, in carrying out the indicator of the test sample for chemical analysis with a fluorescent material Vhen a test sample for chemical analysis is protein, the technique of combining the proteinic amino group and a proteinic luorescent material chemically as a fluorescent material which is common, for example, is combined with the amino roup Fluorescein isothiocyanate () [fluorescein] isothiocyanate, FITC, tetramethylrhodamine isothiocyanate tetramethylrhodamine isothiocyanate, TRITC), etc. are known (the Ono ****, Yuichi Kanaoka, and Fumio Sakiyama -- ne Maeda ****) Proteinic chemical modification (below), Japan Scientific Societies Press, 1981.

0011] On the other hand, quantification becomes sufficiently possible about the sample component separated by this luorescence detecting method also in the above-mentioned capillary tube isoelectric focusing, and the possibility as roteinic super-high sensitivity analytical skill is high by that high separability and detection sensitivity.

0012] However, in isoelectric focusing, since the isoelectric point (pI) is presumed from the static position in the inside f the support for migration of the separated sample, it is required to clarify pH inclination formed in the support for nigration, and the standard marker for it is needed.

0013] A this purpose sake, for example, protein whose pI is clear from the former for example, trypsinogen (pI=9.30) nd lentile lectin (8.00 pI=7.80, 8.20) -- Homo sapiens hemoglobin C (pI=7.50), Homo sapiens hemoglobin A (pI=7.10), horse myoglobin (pI=7.00), Homo sapiens carbo nick anhydrase (pI=6.50), Cow carbo nick anhydrase (pI=6.00), beta actoglobulin B (pI=5.10), Since the fluorescence emitted from these protein itself is very feeble, it is very difficult to use phycocyanin (pI=4.65), amyloglucosidase (pI=3.50), etc. as the above-mentioned marker as it is.

0014] Then, the approach of carrying out the indicator of the protein with which above pI is clear as one approach with a uitable fluorescent material is considered to be the need.

0015] However, since the number and its dissociation condition of the amino group of N-end in protein and the amino roup of a lysine side chain become the big factor which determines proteinic pI (the ******** experiment lecture 2, roteinic chemistry (above), the Japanese Biochemical Society, 1987), if the general indicator approach which carries out hemical bond qualification of the proteinic amino group with a fluorescent material is used, the own isoelectric point of rotein will change a lot.

0016] Furthermore, although the number and location of a fluorescence marker to combine serve as unspecified and it is ne protein as a result since many amino acid in which a fluorescence marker and a reaction are possible exists in protein Since it becomes the mixture in which two or more pI(s) are shown (Flatmark, T., Vesterberg, O., Acta Chem. Scand., 20, 497-1503, 1966), There is a problem that it becomes difficult to clarify correctly pH inclination formed into the support or migration.

0017] Furthermore, proteinic tertiary structure changes with fluorescent material indicators (224 Williamson, A.R., Creth, H.W., Ann.N.Y.Acad.Sci., 209,211- 1973), and own chemical stability of protein worsens. This poses a problem in espect of the preservation stability as a marker.

0018] Therefore, in fluorescence detection isoelectric focusing, pI cannot use beforehand what carried out the indicator
) the clear protein which exists conventionally with the fluorescent material as a marker.

NOTICES *

MEANS

Means for Solving the Problem] In fluorescence detection isoelectric focusing, in order that this invention may clarify orrectly pH inclination formed into the support for migration, by choosing suitable oligopeptide and carrying out an ıdicator with a still more suitable fluorescent material, it shows uniform pI and relates to the marker for fluorescence etection isoelectric focusing moreover characterized by preservation stability being high.

0021] That is, this invention is a marker for fluorescence detection isoelectric focusing used in fluorescence detection ;oelectric focusing, and relates to the marker for fluorescence detection isoelectric focusing characterized by being the ligopeptide containing the cysteine which combined fluorophore coloring matter. The marker for fluorescence detection ;oelectric focusing characterized by for this invention being a marker for fluorescence detection isoelectric focusing used ı fluorescence detection isoelectric focusing, and being the oligopeptide containing at least one cysteine which combined luorophore coloring matter with the sulfhydryl group is offered in more detail.

0022] Moreover, this invention offers the marker for fluorescence detection isoelectric focusing with which said ssociation is characterized by being one chosen from thioester, dithio ester, and the group that consists of thioether ssociation at least.

0023] Furthermore, this invention is amino acid which has the radical in which said oligopeptide emits a proton and has negative charge at least. Amino acid and/of nj individual which set to Kj the acid dissociation constant of the radical /hich emits a proton and has a negative charge Or it is the amino acid which has the radical which receives a proton and as positive charge. The amino acid of ni individual which sets to Ki the acid dissociation constant of the radical which eceives a proton and has positive charge is included. When referred to as Z=sigma i(ni/(1+Ki/[H+]))-sigmaj (nj/(1+ H+]/Kj)) | When filling Z|<0.01 - When log ([H+]) is set to pI, it is offering the marker for fluorescence detection ;oelectric focusing characterized by being 3<pI<10.

0024] Said oligopeptide this invention Moreover, Gly-Cys-Tyr-Lys-Arg, Gly-Cys-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Lys-.ys, Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Tyr-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, Gly-Cys-Glu-Glu or Gly-Cys-Asp-Asp-Asp, and the marker for fluorescence etection isoelectric focusing that crawls and is characterized by being the combination of shoes are offered.

0025] Furthermore, for this invention, said fluorophore coloring matter is a rhodamine and a fluorescein. It is offering ıe marker for fluorescence detection isoelectric focusing characterized by being fluorophore coloring matter chosen from ıe group which consists of cyanine, the India cyanine, the India carbocyanine, the pyronin, RUSHIFA yellow, uinacrine, squaric acid, a coumarin, fluoro ANSE nil maleimide, and an anthracene.

0026] Moreover, this invention offers the marker for fluorescence detection isoelectric focusing with which said luorescence detection isoelectric focusing is characterized by being fluorescence detection capillary tube isoelectric ocusing.

0027] Furthermore, this invention combined fluorophore coloring matter with the sulfhydryl group of a cysteine. Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, The marker kit for fluorescence detection isoelectric focusing which consists of Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu is offered.

0028] Gly-Cys-Tyr-Lys-Arg moreover, this invention combined fluorophore coloring matter with the sulfhydryl group of a cysteine -- Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-

His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu from -- the becoming marker kit for fluorescence detection isoelectric ocusing is offered.
0029] Hereafter, it is based on the gestalt of operation and this invention is explained.
0030]
Embodiment of the Invention]
Oligopeptide) Especially in this invention, oligopeptide is used from various ampholite usable as a marker. In isoelectric ocusing, as a sample usually used, the thing of a principal component has much protein etc., therefore it is because it is lesirable to use the oligopeptide which consists of same dissociable group in order to check pI value of this sample.
0031] However, when oligopeptide is a thing containing not much many amino acid, in case a radical including luorophore is combined, existence of two or more reacting points, mixture of the by-product by the reaction condition, tc. may be produced, a sharp peak with the single marker acquired in this case will be given, and it is not desirable.
0032] Therefore, in this invention, what is necessary is just the oligopeptide which has sufficient amino acid radical for here to be especially no limit in the number of the amino acid which forms oligopeptide, and show the target pI.
0033] The oligopeptide which consists of amino acid as an amphoteric compound has various acidic groups, a basic group and an amino terminal amino group, and a C terminal carboxyl group as an acid-base dissociable group. For example, as an amino acid radical which has an acidic group, it is an aspartic acid, glutamic acid, a cysteine, a thyrosin, etc., and they are a lysine, an arginine, a histidine, etc. as an amino acid radical which has a basic group.
0034] Oligopeptide shows a specific number of charges in a certain pH with these acid-base dissociable groups. Therefore, the number of charges as the whole oligopeptide is able to calculate from the class and number of these acid-base dissociable groups.
0035] For example, it becomes possible to presume the number of charges (Z) as the whole oligopeptide by the following formula.
0036] The amino acid of nj individual which is the amino acid which has the radical which emits a proton and has a negative charge here, and sets to Kj the acid dissociation constant of the radical which emits a proton and has a negative charge, The total number of charges (Z) of the oligopeptide containing the amino acid of ni individual which is the amino acid which has the radical which receives a proton and has positive charge, and sets to Ki the acid dissociation constant of the radical which receives a proton and has positive charge It is given by Z=sigma i(ni/(1+Ki/[H+]))-sigmaj (nj/(1+ H+]/Kj)) (486 a chemical field, 36,470- 1982).
0037] Furthermore, in this formula, -log([H+]) =pH in Z= 0 serves as the isoelectric point pI of that oligopeptide.
0038] Therefore, it becomes possible by using this formula to choose an amino acid radical required for the oligopeptide which shows pI used as the purpose. Moreover, it is required for the variation of a charge to change of pH [in / about the convergency to the isoelectric point / the isoelectric point of a peptide] and dZ/dpH to be large (1484 1479- Shimura, K., and Kasai, K., Electrophoresis, 16, 1995). In order for a dZ/dpH value to become large, it is necessary for a dissociable group with the electric dissociation exponent value near the value of the isoelectric point of the peptide to exist.
0039] Under the present circumstances, it reacts to the halo acetyl derivative of a fluorochrome or a maleimide derivative, and very alternative and high yield, and a labeled compound is generated. As an approach of combining the radical which furthermore contains fluorophore, when combining with the sulfhydryl group of the cysteine of this oligopeptide, basic dissociation by the amino terminal amino group will also be demonstrated, and the convergent good indicator peptide which has pI before and after pH7.6 which is electric dissociation exponent of the amino terminal amino group will be obtained.
0040] In order to enable this presumption and to choose a required amino acid radical, as an electric dissociation exponent which the dissociable group of the above-mentioned acid-base nature has For example, alpha-carboxyl (C erminal) (3.6), beta-carboxyl (Asp) (3.95), gamma-carboxyl (Glu) (4.45), an imidazole (His) (6.45), alpha-amino (amino erminal) (7.6), a thiol (Cys) (8.5), phenol nature hydroxy (Tyr) (9.8), epsilon-amino (Lys) (10.2), a GUANIJIUMU radical (Arg) (12.5), etc. are usable.
0041] For example, in order to make pI small, to choose an aspartic acid or glutamic acid and to enlarge pI, the thing which choose an arginine or a lysine and for which alpha-amino group is made free is also still more possible. What is necessary is similarly, just to choose a thyrosin and a histidine, in order to carry out pI among them.
0042] Especially in this invention, it is three or more as range of pI, and 11 or less thing is desirable. It is three or more further especially especially as range of pI, and ten or less thing is desirable.
[0043] Furthermore, in this invention, it is not restricted especially about the synthetic approach of the oligopeptide

containing the amino acid chosen desirably. The composition (the foundation of peptide synthesis, such as a liquid phase process and a solid phase technique, an experiment, Nobuo Izumiya, Tetsuo Kato, the Aoyagi ****, the near Michinori work, Maruzen 1985) by the general synthetic approach or composition by the automatic composition approach is preferably usable.

[0044] Moreover, in this invention, a required amino acid radical exists, and if a labile point is still more controllable, the all or a part is suitably usable [the oligopeptide containing at least one cysteine of the natural product origin]. Specifically, oxytocin, vasopressin, the oligopeptide of the vasotocin origin, and the oligopeptide of the somatostatin origin are mentioned.

[0045] It is also possible to compound the oligopeptide which combines an amino acid radical still more nearly required for the oligopeptide of the natural product origin by chemosynthesis, and has a desirable amino acid radical. Under the present circumstances, there is especially no limit in the number of cysteines, or the array location of a cysteine.

[0046] (Fluorophore) What is necessary is not to be restricted especially about usable fluorophore's class, but just to include the fluorescence coloring matter generally known in this invention.

[0047] For example, a rhodamine, a fluorescein What is necessary is just cyanine, the India cyanine, the India carbocyanine, the pyronin, RUSHIFA yellow, quinacrine, squaric acid, a coumarin, fluoro ANSE nil maleimide, etc. In this invention, a rhodamine, cyanine dye, etc. are especially desirable and it is usable.

[0048] It is desirable to introduce rhodamine fluorophore (Handbook of Fluorescent Probes and Research Chemicals, 5th Edition MOLECULAR PROBES, INC., 1992) especially in this invention.

[0049] This rhodamine fluorophore has the fluorescence which has absorption maximum (molar extinction coefficient 93,000) in 556nm in a methanol, and makes 576nm the maximum.

[0050] Furthermore in this invention, there is an aromatic heterocyclic compound or polycyclic aromatic hydrocarbon as usable fluorophore suitably (for example, fluorescence phosphorimetry, Taiji Nishikawa, Keizo Hiraki work, KYORITSU SHUPPAN, 1989 reference). For example, those derivatives, such as an anthracene, naphthalene, a phenanthrene, a quinoline, a pyrene, and perylene, are especially suitably usable. It becomes such hydrocarbon fluorophores combinable with oligopeptide suitably by preparing a suitable joint radical.

[0051] (Joint radical) In this invention, there is especially no limit in the class of joint radical which combines a fluorophore radical and the sulfhydryl group of the cysteine of oligopeptide. The radical which contains fluorophore through a joint radical may combine with said sulfhydryl group, and may join together through a suitable joint radical.

[0052] In this case, it is desirable that a radical including fluorophore combines with said sulfhydryl group by for example, thioester association, the dithio ester bond, thioether association (sulfide association), etc. Especially in this invention, thioether association is desirable.

[0053] The introductory approach of a (fluorophore dye base)-(joint radical)-S-cysteine joint radical does not have especially a limit. When the radical containing fluorophore coloring matter has a halogenation methyl group (for example, an iodine methyl group, -CH2I), an activity ester group, an acid chloride, an acid anhydride, a maleimide radical, etc., these active groups and reactions are possible for it, and 2 functionality radical in which the sulfhydryl group of a cysteine and a reaction are still more possible is desirable.

[0054] For example, when a rhodamine fluorophore radical has an acid-chloride radical (for example, coloring matter-SO2Cl) Thioamide association (coloring matter-SO2NH(CH2)2NH2) is first formed by the reaction with diamine (for example, ethylenediamine). Making it react with monoiodoacetic acid N-malic acid imide ester furthermore, coloring matter-SO2NH(CH2)2NHCOCH2I obtained reacts with the sulfhydryl group of the cysteine of oligopeptide alternatively easily, and forms thioether association.

[0055] Coloring matter - An SO2NH(CH2)2NHCOCH2-S-cysteine book reaction can be carried out on mild conditions. and does not give change to other oligopeptide parts and fluorochrome sections.

[0056] Furthermore, since a reaction is made possible only at said sulfhydryl group when two or more labile points are in oligopeptide, selection of a reaction condition, a reaction reagent, etc. is possible. Since this selection is enabled, the technique of general organic synthesis is suitably usable.

[0057] For example, if required, it is one of the desirable technique to protect beforehand other reactant substituents (for example, the amino group, a phenolic group, etc.).

[0058] In this invention, the purity and preservation stability of the acquired marker for fluorescence detection capillary tube isoelectric focusing can be checked with isoelectric focusing.

[0059] If still more nearly required, it can refine simple using high performance chromatography. Efficient separation

urification is attained by using an opposition system column, especially the bulking agent to which the chemical bond of ıe octadecyl radical was carried out in that case. It is usable wavelength suitable [280nm] for detection.
0060] (pI measurement) In this invention, pI of the marker for fluorescence isoelectric focusing is measurable erforming isoelectric focusing and measuring mobility with the commercial marker for isoelectric focusing, or by ıeasuring directly pH of the migration location of an indicator peptide.
0061] Slab (plane) gel electrofocusing which uses polyacrylamide as a base material is desirable, and isoelectric focusing ; usable (Righetti, P.G., Isoelectric Focusing:Theory, Methodology and Applications, Elsevier, Amsterdam, 1983 eference).
0062] The detection by the dyeing approach is suitably usable in a general approach (for example, Coomassie Brilliant Blue-R250 dyeing).
0063] Furthermore, the detection by fluorometry is suitably usable in a general approach, for example, the approach by ıe densitometer, (for example, the Shimadzu make, Shimazu two-wave premature start spot scanning densitometer CS9300PC).
0064] (Capillary tube isoelectric focusing) There is especially no limit in the equipment for capillary tube isoelectric ocusing which can use the indicator oligopeptide concerning this invention as a marker.
0065] Generally, with capillary tube isoelectric-focusing equipment, the peptide which carried out the indicator of the luorescent material is condensed by the location of pH formed in the support for migration according to each pI, and tands it still. Furthermore, these locations may be checked by detecting the fluorescence generated by excitation light xposure.
0066] In this invention, although especially the excitation light source is not restricted, especially its use of the laser vhich enables the stable optical exposure is desirable.
0067] For example, in this invention, helium neon laser is suitably usable as an excitation light to the rhodamine coloring natter which is fluorophore.
0068] The gradient of pH formed in the support for migration by the fluorescence detector formed in the end of a apillary tube can be checked.
0069] As explained in the top, the marker for fluorescence detection isoelectric focusing of this invention is compounded y combining the radical which has the fluorophore of oligopeptide, and has stability uniform [pI] and high moreover. Therefore, in fluorescence detection isoelectric point migration, it becomes a marker for the high sensitivity analysis at he time of checking the isoelectric point of the sample matter which migrated based on the gradient of pH formed in the upport for migration.
0070] Therefore, the marker for fluorescence detection isoelectric focusing of this invention becomes usable also to the apillary tube isoelectric point migration approach.
0071] Hereafter, although this invention is explained still more concretely based on an example, this invention is not imited to the following examples, unless the summary is exceeded.
0072] (Marker kit for fluorescence detection isoelectric focusing) As explanation was given [above-mentioned], oligopeptide usable to the marker for fluorescence isoelectric focusing concerning this invention combines [choose imely and] and is usable.
0073] That is, the marker concerning this invention shows the good focus nature which is what shows good coincidence of calculated value and an actual measurement over the range of 3-10 as pI (specifically mentioned to Table 1). Furthermore, the marker concerning this invention is the oligopeptide of low molecular weight, and even if it doubles two or more sets of them and uses it under various isoelectric-focusing conditions, the focusing of it is independently carried out to each pI value, respectively. Based on the above-mentioned table 1, the suitable thing for which number selection is nade becomes possible about the marker concerning this invention which is in within the limits to specifically use as a narker as the above-mentioned combination.
0074] for example, the number shown all over Table 1 -- ten kinds of combination (Gly-Cys-Glu-Tyr-Tyr-Lys-Lys) of 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu are one example of a desirable kit. These cover pI of the large range and the pI also serves as abbreviation regular ntervals.
0075] moreover -- the same -- 12 kinds of combination (Gly-Cys-Tyr-Lys-Arg) of 1, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-

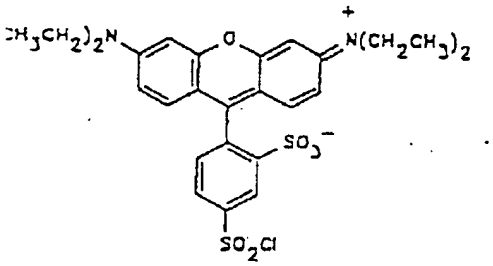
Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, and Gly-Cys-Glu-Glu are also one example of a desirable kit.

Translation done.]

:XAMPLE

Example]
Composition of the marker for fluorescence detection isoelectric focusing)
1) Gly-Cys-Tyr-Lys-Arg, Gly-Cys-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Lys-Lys, Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys, Gly-Cys-'yr-Lys, Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, ily-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, Gly-'ys-Glu-Glu, preparation of Gly-Cys-Asp-Asp-Asp.
0077] a peptide -- the peptide automatic synthesizer unit made from Applied Biosystems -- Boc -- it compounded based n law (see the new chemistry experiment lecture 1, protein VI, the edited by Japanese Biochemical Society, and 1992), nd the high-speed liquid chromatograph refined the obtained oligopeptide. The conditions used for the high-speed liquid hromatograph are column [by YMC CO., LTD.] S-5,120A. The rate of flow used a part for /, and 20ml detection vavelength for 220nm and a mobile phase on the following gradient / 80-minute conditions with -0.1% trifluoroacetic cid of acetonitrile inclination using ODS (30mm bore x250mm overall length).
0078]
)ligopeptide HPLC mobile phase inclination conditions ---------- Gly-Cys-Tyr-.ys-Arg 3%- 23%Gly-Cys-Tyr-Lys-Lys 4.5% - 24.5%Gly-Cys-Tyr-Tyr-Lys-Lys 2.5% - 22.5%Gly-Cys-Tyr-Tyr-Tyr-.ys-Lys 1%- 19%Gly-Cys-Tyr-Lys 3.7% - 23.7%Gly-Cys-Glu-Tyr-Tyr-Lys-Lys 2.3% - 22.3%Gly-Cys-Glu-His-His-Iis-Arg 1%- 18%Gly-Cys-Glu-His-Arg 0% - 5%Gly-Cys-Glu-His-His 0.1%- 10%Gly-Cys-Glu-Arg 1%- 10%Gly-Cys-;lu-His0%- 5%Gly-Cys-Asp-Asp-His-His 1%- 15%Gly-Cys-Glu-Glu-His 1%- 13%Gly-Cys-Asp-Asp-Arg 1% - 18.5% ;ly-Cys-Glu-Glu 1%- 19%Gly-Cys-Asp-Asp-Asp 0.5%- 10% ----------
0079] (2) Preparation of lisamine rhodamine B (LRB) iodoacetamide (Alexander, H., Lewis, A.S., and Ram, R.C., .Medicinal Chem., 1976, Vol19, 1279-1283)
a) The 10mg lisamine rhodamine B sulfonyl chloride (structure is shown made in [molecular PUROBUSU] the U.S. nd in the following) dissolved in 0.3ml dimethylformamide at mixture (preparation ethylenediamine 104mg of a LRB .mine and dimethylformamide 0.4ml) was dropped, and it was made to react at a dark place room temperature for 1 hour.

0080]
Formula 1]



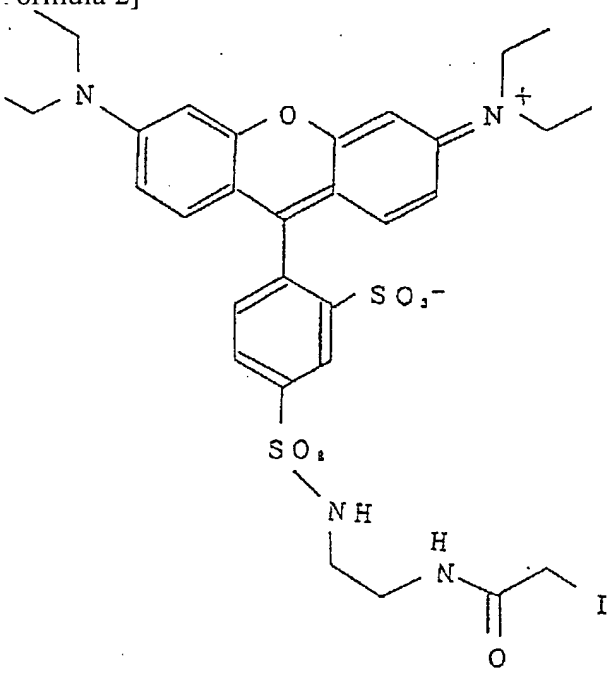
0081] After carrying out evaporation to dryness of the reaction mixture under reduced pressure, it dissolved in 2ml of ormic-acid solutions 20% acetonitrile-1%, and the whole-quantity style was carried out to Dowex-1 (H1 mold) 1ml :olumn which equilibrated with the formic-acid solution 20% acetonitrile-1%, and bypassing fractions were collected,

vaporation to dryness was carried out under reduced pressure, and it considered as the crude material of a LRB amine. pectrometry determined the concentration, having dissolved this in acetonitrile 0.4ml and 1.6ml (pH7.5) of 0.1M sodium hosphate buffer solutions, and having used the molar extinction coefficient in 570nm as 93,000. It applied to the pposition chromatograph column (TOSOH ODS-80Ts, the diameter of 4.6mm, die length of 25cm) which equilibrated .RB amine crude-material 150nmol with the trifluoroacetic acid solution 25% acetonitrile-0.1%, and it was eluted over 0 minutes, having applied the concentration straight-line inclination of 25-55% of acetonitrile, and detected by acting as ıe monitor of the 280nm absorption. The biggest peak was isolated preparatively (127nmol) and it used for the following ctuation as a purification LRB amine.

)082] (b) it hardened by drying under reduced pressure of the fraction prepared by iodine acetylation (a) of a LRB mine, and it dissolved in 88micro of 1:4 mixed liquor l of an acetonitrile and the 0.1M sodium phosphate buffer solution)H7.5) after that, 12micro of dimethylformamide solutions l of 10mM monoiodoacetic acid N-malic acid imide ester 'as added to this, and it was made to react at a room temperature for 1 hour It applied to the opposition chromatograph n the same conditions which explained the whole quantity of reaction mixture by (a), the eluted main peak was isolated reparatively as LRB-iodoacetamide (70nmol), and it used for the indicator of a peptide. The structure of LRB-)doacetamide was shown below.

)083]

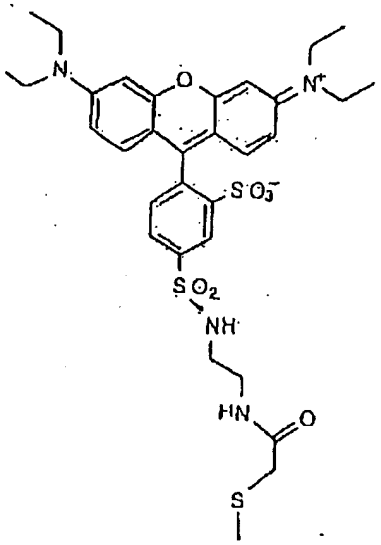
Formula 2]



)084] (3) The column solution containing purification coloring matter (LRB-iodoacetamide) 25nmol prepared with the ıdicator (2) of a peptide was hardened by drying in the test tube, and it dissolved in acetonitrile 20microl after that. 0micro (concentration 5mM, 50nmol) of peptide water solutions l and the buffer solution (0.1 M Na-Pi, 5mM EDTA,)H7.5) which were prepared by (1) were added to this, and it was made to react to it at a room temperature in a dark place)vernight. reaction mixture was applied to the opposition chromatograph column (TOSOH ODS-80Ts, the diameter of .6mm, die length of 25cm) which equilibrated with the trifluoroacetic acid solution 25% acetonitrile-0.1%, and the ıdicator peptide was eluted in the 1ml rate of flow for /, having applied the concentration straight-line inclination of 25- 5% of acetonitrile over 30 minutes -- it isolated preparatively. Detection was performed by 280nm absorption. The yield)f an indicator peptide was 80%. The concentration of an indicator peptide set the molar extinction coefficient in 570nm o 93,000, and determined it by spectrometry. The structure of oligopeptide Gly-Cys-Glu-Tyr-Tyr-Lys-Lys by which the ndicator was carried out to below is shown.

0085]

Formula 3]



H-Gly-Cys-Glu-Tyr-Tyr-Lys-Lys-OH

0086] Gly-Cys-Tyr-Lys, (pI of a fluorescent material indicator peptide) Gly-Cys-Glu-Tyr-Tyr-Lys-Lys, Gly-Cys-Glu-His-His-His-Arg, Gly-Cys-Glu-His-Arg, Gly-Cys-Glu-His-His, Gly-Cys-Glu-Arg, Gly-Cys-Glu-His, Gly-Cys-Asp-Asp-His-His, Gly-Cys-Glu-Glu-His, Gly-Cys-Asp-Asp-Arg, Gly-Cys-Glu-Glu, and the above-mentioned fluorescent material ndicator oligopeptide of Gly-Cys-Asp-Asp-Asp

NOTICES *

.This document has been translated by computer. So the translation may not reflect the original precisely.
.**** shows the word which can not be translated.
.In the drawings, any words are not translated.

DESCRIPTION OF DRAWINGS

Brief Description of the Drawings]
Drawing 1] It is the electrophoresis photograph in which the zymogram at the time of performing **** electrophoresis of the marker for isoelectric focusing of 16 kinds of fluorescent material indicator peptides concerning this invention and marketing is shown. The numbers 1-16 in drawing correspond to the oligopeptide shown by numbers 1-16 all over Table .

Drawing 2] It is drawing showing the transit time and fluorescence intensity at the time of performing fluorescence detection capillary tube isoelectric focusing of the fluorescent material indicator peptide concerning this invention.

Translation done.]

NOTICES *

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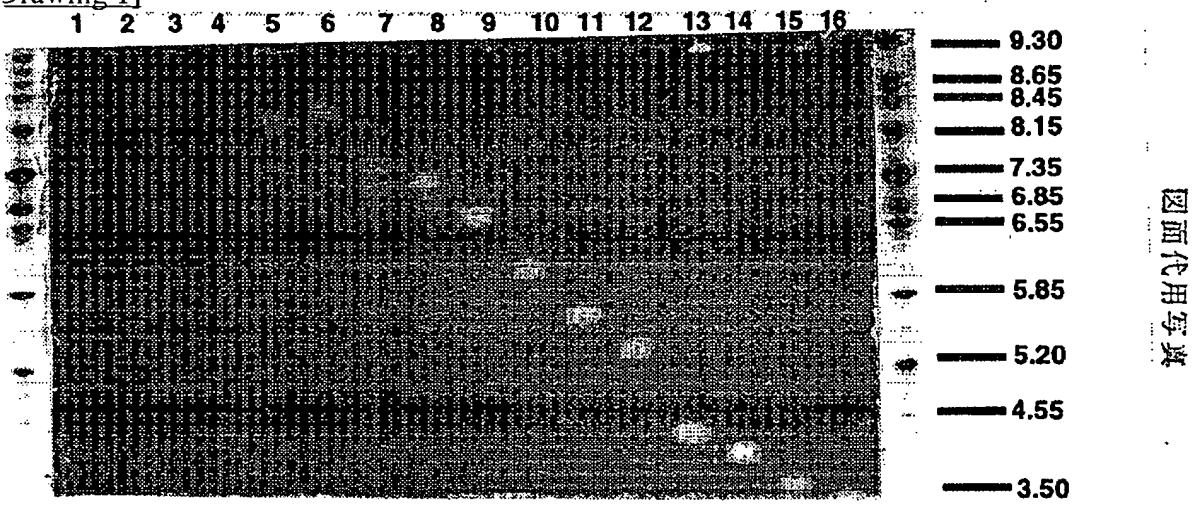
.In the drawings, any words are not translated.

)RAWINGS

Drawing 1]



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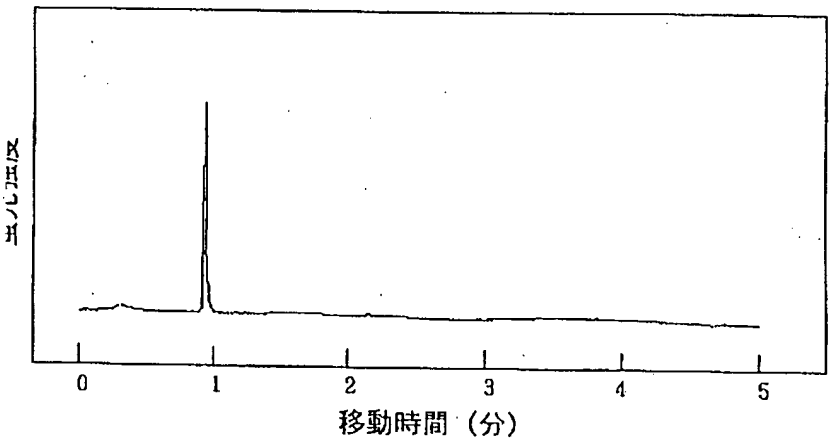

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(19)日本国特許庁（ＪＰ）　(12)公開特許公報（Ａ）　(1

特開平10－197481

(43)公開日　平成10年(1998)７月31日

(51)Int.Cl.⁶		識別記号	ＦＩ		
Ｇ０１Ｎ	27/447		Ｇ０１Ｎ	27/26	３０１Ｂ
Ｃ０７Ｋ	1/13		Ｃ０７Ｋ	1/13	
	5/103			5/103	
	7/06	ＺＮＡ		7/06	ＺＮＡ
			Ｇ０１Ｎ	27/26	３２５Ａ

審査請求　未請求　請求項の数８　ＦＤ　（全 13 頁）

(21)出願番号　特願平9－267974

(22)出願日　平成９年(1997)９月12日

(31)優先権主張番号　特願平8－318762

(32)優先日　平８(1996)11月14日

(33)優先権主張国　日本（ＪＰ）

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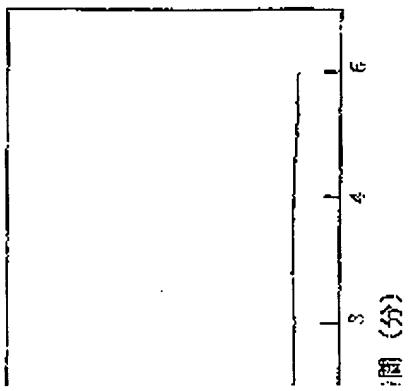
最終頁に続く

(54)【発明の名称】　蛍光検出等電点電気泳動用マーカー

(57)【要約】

【課題】　本発明は、蛍光検出等電点電気泳動法において、試料物質の等電点を確認するため使用される蛍光検出等電点電気泳動用マーカーを提供するものである。

【解決手段】　本発明により、オリゴペプチドのシステインのＳＨ基に発蛍光団色素を設けることにより、ｐｉの大きな蛍光物質標識オリゴペプチドが得られる。この際、オリゴペプチドと発蛍光団は温和な条件下で結合され得るため、オリゴペプチドに結合させる蛍光物質の数と位置が制御され、従って得られる蛍光検出等電点電気



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【特許請求の範囲】

【請求項１】　蛍光検出等電点電気泳動法において用いる蛍光検出等電点電気泳動用マーカーであって．ＳＨ基に発蛍光団色素を結合したシステインを少なくとも１つ含むオリゴペプチドであることを特徴とする蛍光検出等電点電気泳動用マーカー。

【請求項２】　前記結合が．少なくとも、チオエステル．ジチオエステル、チオエーテル結合からなる群から選ばれる１つであることを特徴とする請求項１に記載の蛍光検出等電点電気泳動用マーカー。

【請求項３】　前記オリゴペプチドが．少なくとも、プロトンを放出して負電荷を持つ基を有するアミノ酸であって．プロトンを放出して負電荷を持つ基の酸解離定数をK_1とするn_1個のアミノ酸および／または、プロトンを受け取って正電荷を持つ基を有するアミノ酸であって．プロトンを受け取って正電荷を持つ基の酸解離定数をK_2とするn_2個のアミノ酸とを含み．$Z=\Sigma_1(n_1/(1+K_1/[H^+]))-\Sigma_2(n_2/(1+[H^+]/K_2))$とした際に．$|Z|<0.01$をみたす際の$-\log([H^+])$をｐｉとした場合、$3<pi<10$であることを特徴とする．請求項１又は２のいずれかの請求項に記載の蛍光検出等電点電気泳動用マーカー。

【請求項４】　前記オリゴペプチドが．Gly-Cys-Tyr-Lys-Arg、Gly-Cys-Tyr-Lys-Lys、Gly-Cys-Tyr-Tyr-Lys-Lys、Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys、Gly-Cys-Tyr-Lys．Gly-Cys-Glu-Tyr-Tyr-Lys-Lys、Gly-Cys-Glu-His-His-His-Arg、Gly-Cys-Glu-His-Arg、Gly-Cys-Glu-His-His．Gly-Cys-Glu-Arg．Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His、Gly-Cys-Glu-Glu-His．Gly-Cys-Asp-Asp-Arg．Gly-Cys-Glu-Glu-His、Gly-Cys-Glu-Glu-Arg、Gly-Cys-Asp-Asp-Asp からなる群から選ばれる少なくとも１つであることを特徴とする請求項１～３のいずれかの請求項に記載の蛍光検出等電点電気泳動用マーカー。

【請求項５】　前記発蛍光団色素が．ローダミン．フルオレセイン 、シアニン、インドシアニン．インドカルボシアニン、ピロニン、ルシファーイエロー、キナクリン．スクエア酸．クマリン．フルオロアンセニルマレイミド．アントラセンからなる群より選ばれる発蛍光団色素であることを特徴とする請求項１～４のいずれかの請求項に記載の蛍光検出等電点電気泳動用マーカー。

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【請求項６】　システインのＳＨ基に発蛍光団色素を結合した．Gly-Cys-Tyr-Lys-Arg、Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys．Gly-Cys-Glu-Tyr-Tyr-Lys-Lys、Gly-Cys-Glu-His-His-His-Arg．Gly-Cys-Glu-His-Arg、Gly-Cys-Glu-His-His．Gly-Cys-Glu-Arg．Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His、Gly-Cys-Glu-Glu-His．Gly-Cys-Asp-Asp-Arg、Gly-Cys-Glu-Glu からなる蛍光検出等電点電気泳動用マーカーキット。

【請求項７】　システインのＳＨ基に発蛍光団色素を結合した．Gly-Cys-Tyr-Lys-Arg、Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys．Gly-Cys-Glu-Tyr-Tyr-Lys-Lys、Gly-Cys-Glu-His-His-His-Arg．Gly-Cys-Glu-His-Arg、Gly-Cys-Glu-His-His．Gly-Cys-Glu-Arg．Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His、Gly-Cys-Glu-Glu-His．Gly-Cys-Asp-Asp-Arg、Gly-Cys-Glu-Glu からなる蛍光検出等電点電気泳動用マーカーキット。

【請求項８】　システインのＳＨ基に発蛍光団色素を結合した．Gly-Cys-Tyr-Lys-Arg、Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys．Gly-Cys-Glu-Tyr-Tyr-Lys-Lys、Gly-Cys-Glu-His-His-His-Arg．Gly-Cys-Glu-His-Arg、Gly-Cys-Glu-His-His．Gly-Cys-Glu-Arg．Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His、Gly-Cys-Glu-Glu-His．Gly-Cys-Asp-Asp-Arg、Gly-Cys-Glu-Glu からなる蛍光検出等電点電気泳動用マーカーキット。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は．蛍光検出等電点電気泳動法において使用する蛍光検出等電点電気泳動用マーカーおよびマーカーキットに関する。

【０００２】

【従来の技術】分子の表面に電荷を有する粒子を電解質溶液に浮遊させて電流を通ずると、それぞれの荷電と反対の方向に向かって粒子が移動する現象が、１８９２年にＰｉｃｔｏｎ．Ｈ．とＬｉｎｄｅｒ．Ｓ．Ｅ．によって観察されて以来、電気泳動の現象は物質の分離分析の手段として用いられるようになり、１９３７年にＴｉｓｅｌｉｕｓが装置を完成させてからは．今日まで急速にその応用面が拡大されてきている。

【０００３】一般の電気泳動が、ある特定のｐＨにおける荷電状態の差を利用しているのに対し、１９６６年にＶｅｓｔｅｒｂｅｒｇ，Ｏ．とＳｖｅｎｓｓｏｎ．Ｈ．が合成両性電解質であるアンフォラインを開発してからは．それぞれの両性電解質がある値のｐＨにおいて、その実効電荷がゼロとなり．泳動しなくなる現象を利用した等電点電気泳動法が確立された。

【０００４】ここで実効電荷がゼロとなるようなｐＨの値をその物質の等電点という。等電点電気泳動法では、試料は泳動用担体に形成されたｐＨ勾配において．その等電点に等しい位置に濃縮されて静止する。

【０００５】このように．試料が焦点的に濃縮されて分離されるので．非常に高い分離能を持つ電気泳動法として．物質の分離・分析に利用されている。

【０００６】また、近年．電気泳動を内径５～１００μｍ．長さが３０～１００ｃｍの溶融シリカでできた毛細管（キャピラリー）の中で行なうキャピラリー電気泳動

ら検出する方法（紫外可視検出法）が一般的である。

【0009】さらに高感度な検出方法として、あらかじめ分析試料を蛍光物質で標識し、分離されてきた試料成分に光を照射し、発せられる蛍光を検出することにより濃度を定量化する検出方法（蛍光検出法）が考えられる。

【0010】このために分析試料を蛍光物質で標識するにあたっては、分析試料がタンパク質の場合、タンパク質のアミノ基と蛍光物質とを化学的に結合させる手法が一般的であり、例えば、アミノ基に結合させる蛍光物質として、フルオレセインイソチオシアネート（fluorescein isothiocyanate, FITC）やテトラメチルローダミンイソチオシアネート（tetramethylrhodamine isothiocyanate, TRITC）等が知られている（大野素徳、金岡祐一、崎山文夫、前田浩、蛋白質の化学修飾（下）、学会出版センター、1981）。

【0011】一方、上記のキャピラリー等電点電気泳動法においてもこの蛍光検出法により分離された試料成分を十分定量化可能となり、その高い分離能と検出感度により、タンパク質の超高感度分析技術としての可能性が高い。

【0012】しかしながら、等電点電気泳動においては、分離された試料の泳動用担体中での静止位置から等電点（pI）が決定されるものであるから、泳動用担体に形成されたpH勾配を明確にしておくことが必要であり、このための標準マーカーが必要となる。

【0013】この目的のため、例えば従来からpIが明らかとなっているタンパク質（例えば、トリプシノーゲン（pI＝9.30）、レンチルレクチン（pI＝7.80、8.00、8.20）、ヒト・ヘモグロビンC（pI＝7.50）、ヒト・ヘモグロビンA（pI＝7.10）、ウマ・ミオグロビン（pI＝7.00）、ヒト・カーボニックアンヒドラーゼ（pI＝6.50）、ウシ・カーボニックアンヒドラーゼ（pI＝6.00）、β－ラクトグロブリンB（pI＝5.10）、フィコシアニン（pI＝4.65）、アミログルコシダーゼ（pI＝3.50）等をそのまま上記マーカーとして用いることは、これらのタンパク質自身から発せられる蛍光が非常に微弱なため極めて困難である。

【0016】さらに、タンパク質には蛍光標識物質と反応可能なアミノ酸が多く存在するため、結合する蛍光標識物質の数と位置が不特定となり、結果として、一つのタンパク質であるが、複数のpIを示す混合物となってしまうため（Flatmark, T., Vesterberg, O., Acta Chem. Scand., 20, 1497－1503, 1966）、泳動用担体中に形成されたpH勾配を正確に明らかにすることが困難となるという問題がある。

【0017】さらに、蛍光物質標識によりタンパク質の3次構造が変化し（Williamson, A. R., Kreth, H. W., Ann. N. Y. Acad. Sci., 209, 211－224, 1973）、タンパク質自身の化学的安定性が悪くなる。このことは、マーカーとしての保存安定性の点で問題となる。

【0018】従って、蛍光検出等電点電気泳動において、従来よりあるあらかじめpIが明らかなタンパク質に蛍光物質で標識したものをマーカーとして用いることはできない。

【0019】本発明者等は、上記問題点を解決するため鋭意研究し、発蛍光団色素を有したシステインを含むオリゴペプチドが、単一特有のpIを示し、しかも大きなpI値（3＜pI＜10）の範囲をカバーし、さらに保存安定性にも優れていることを見いだし、本発明を完成するにいたった。

【0020】

【課題を解決するための手段】本発明は、蛍光検出等電点電気泳動において、泳動用担体中に形成されたpH勾配を正確に明らかにするため、適当なオリゴペプチドを選択し、さらに適当な蛍光物質で標識することにより、均一なpIを示し、しかも保存安定性が高いことを特徴とする蛍光検出等電点電気泳動用マーカーに係るものである。

【0021】すなわち、本発明は、蛍光検出等電点電気泳動法において用いる蛍光検出等電点電気泳動用マーカーであって、発蛍光団色素を結合したシステインを含むオリゴペプチドであることを特徴とする蛍光検出等電点電気泳動用マーカーに係るものである。より詳しくは、本発明は、蛍光検出等電点電気泳動法において用いる蛍光検出等電点電気泳動用マーカーであって、SH基に発

有するアミノ酸であって．プロトンを放出して負電荷を持つ基の酸解離定数をK_1とするn₁個のアミノ酸および／または、プロトンを受け取って正電荷を持つ基を有するアミノ酸であって、プロトンを受け取って正電荷を持つ基の酸解離定数をK_1とするn₁個のアミノ酸とを含み．$Z=\Sigma_1(n_1/(1+K_1/[H^+]))-\Sigma_1(n_1/(1+[H^+]/K_1))$とした際に．$|Z|<0.01$をみたす際の$-\log([H^+])$をｐＩとした場合．$3<pI<10$であることを特徴とする蛍光検出等電点電気泳動用マーカーを提供することである。

【００２４】また、本発明は、前記オリゴペプチドが、Gly-Cys-Tyr-Lys-Arg. Gly-Cys-Tyr-Lys-Lys、Gly-Cys-Tyr-Tyr-Lys-Lys、Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys、Gly-Cys-Tyr-Lys. Gly-Cys-Glu-Tyr-Tyr-Lys-Lys. Gly-Cys-Glu-His-His-His-Arg. Gly-Cys-Glu-His-Arg. Gly-Cys-Glu-His-His、Gly-Cys-Glu-Arg、Gly-Cys-Glu-His. Gly-Cys-Asp-Asp-His-His. Gly-Cys-Glu-Glu-His、Gly-Cys-Asp-Asp-Arg、Gly-Cys-Glu-Glu. Gly-Cys-Asp-Asp-Aspのいずれか、またはいくつかの組合せであることを特徴とする蛍光検出等電点電気泳動用マーカーを提供するものである。

【００２５】さらに、本発明は、前記発蛍光団色素が、ローダミン、フルオレセイン．シアニン．インドシアニン．インドカルボシアニン、ピロニン、ルシファーイエロー、キナクリン、スクエア酸、クマリン、フルオロアンセニルマレイミド、アントラセンからなる群より選ばれる発蛍光団色素であることを特徴とする蛍光検出等電点電気泳動用マーカーを提供することである。

【００２６】また、本発明は、前記蛍光検出等電点電気泳動法が、蛍光検出キャピラリー等電点電気泳動法であることを特徴とする蛍光検出等電点電気泳動用マーカーを提供するものである。

【００２７】さらに、本発明は、システインのＳＨ基に発蛍光団色素を結合した．Gly-Cys-Glu-Tyr-Tyr-Lys-Lys. Gly-Cys-Glu-His-His-His-Arg. Gly-Cys-Glu-His-Arg. Gly-Cys-Glu-His-His、Gly-Cys-Glu-Arg、Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His. Gly-Cys-Glu-Glu-His、Gly-Cys-Asp-Asp-Arg. Gly-Cys-Glu-Gluからなる蛍光検出等電点電気泳動用マーカーキットを提供するものである。

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【００３０】

【発明の実施の形態】

（オリゴペプチド）本発明においては．マーカーとして使用可能な種々の両性物質から、特にオリゴペプチドを使用する。等電点電気泳動法において．通常使用される試料としては．タンパク質等が主成分のものが多く、従って該試料のｐＩ値を確認するためには、同様な解離基からなるオリゴペプチドを用いることが好ましいからである。

【００３１】しかしながら．オリゴペプチドがあまり多くのアミノ酸を含むものである場合においては．発蛍光団を含む基を結合する際に．複数の反応点の存在や、反応条件による副生成物の混在などを生じる可能性があり．この場合．得られるマーカーが単一の鋭いピークを与えないこととなり好ましくない。

【００３２】従って、本発明においては、オリゴペプチドを形成するアミノ酸の数には特に制限はなく．目的のｐＩを示すに十分なアミノ酸基を有するオリゴペプチドであればよい。

【００３３】両性化合物としてのアミノ酸からなるオリゴペプチドは．酸塩基解離基として種々の酸性基．および塩基性基、そしてＮ末端アミノ基．Ｃ末端カルボキシル基を有する。例えば酸性基を有するアミノ酸基としては．アスパラギン酸、グルタミン酸．システイン．チロシン等であり．また塩基性基を有するアミノ酸基としてはリシン、アルギニン、ヒスチジン等である。

【００３４】これらの酸塩基解離基によりオリゴペプチドはあるｐＨにおいて特定の荷電数を示す。従って、これらの酸塩基解離基の種類と数から．オリゴペプチド全体としての荷電数が計算することが可能である。

【００３５】例えば、次の式によりオリゴペプチド全体としての荷電数（Ｚ）を推定することが可能となる。

【００３６】ここで、プロトンを放出して負電荷を持つ基を有するアミノ酸であって、プロトンを放出して負電荷を持つ基の酸解離定数をK_1とするn₁個のアミノ酸と．プロトンを受け取って正電荷を持つ基を有するアミノ酸であって．プロトンを受け取って正電荷を持つ基の酸解離定数をK_1とするn₁個のアミノ酸とを含むオリゴペプチドの全荷電数（Ｚ）は．$Z=\Sigma_1(n_1/(1+K$

に対する荷電の変化量、dZ／dpHが大きいことが必要である（Shimura,K., and Kasai,K.,Electrophoresis,16,1479-1484,1995）。dZ／dpH値が大きくなるためには、そのペプチドの等電点の値に近いpKa値を持つ解離基が存在することが必要となる。

【0039】この際、蛍光色素のハロアセチル誘導体又はマレイミド誘導体と極めて選択的かつ高収率に反応し、標識化合物を生成する。さらに発蛍光団を含む基を結合する方法として、該オリゴペプチドのシステインのSH基に結合する場合においては、N末端アミノ基による塩基性解離も発揮され、N末端アミノ基のpKaであるpH7．6前後にpIを有する収束性の良い標識ペプチドが得られることとなる。

【0040】この推定を可能とし、必要なアミノ酸基を選択するために、上記の酸塩基性の解離基の有するpKaとして、例えば、α－カルボキシル（C末端）（3．6）、β－カルボキシル（Asp）（3．95）、γ－カルボキシル（Glu）（4．45）、イミダゾール（His）（6．45）、α－アミノ（N末端）（7．6）、チオール（Cys）（8．5）、フェノール性ヒドロキシ（Tyr）（9．8）、ε－アミノ（Lys）（10．2）、グアニジウム基（Arg）（12．5）等が使用可能である。

【0041】例えば、pIを小さくするためには、アスパラギン酸、またはグルタミン酸を選択し、pIを大きくするには、アルギニン、またはリシンを選択する、さらに、α－アミノ基をフリーにすることも可能である。同様に、pIをそれらの間にするためには、チロシンやヒスチジンを選択すればよい。

【0042】本発明においては、pIの範囲として特に3以上であって、11以下のものが好ましい。さらに特に、pIの範囲として特に3以上であって、10以下のものが好ましい。

【0043】さらに、本発明においては、望ましく選択されたアミノ酸を含むオリゴペプチドの合成方法については、特に制限されない。一般的な合成方法による合成（液相法、固相法等、ペプチド合成の基礎と実験、泉谷信夫、加藤哲夫、青柳泉彦、鵜飼典著、丸善1985年）、または自動合成方法による合成等が好ましく使用可能である。

る。この際、システイン残又はシステインの配列位置には特に制限はない。

【0046】（発蛍光団）本発明において使用可能な発蛍光団の種類については特に制限されず、一般に知られている蛍光性色素を含むものであればよい。

【0047】例えば、ローダミン、フルオレセイン、シアニン、インドシアニン、インドカルボシアニン、ピロニン、ルシファーイエロー、キナクリン、スクエア酸、クマリン、フルオロアンセニルマレイミド等であればよい。本発明においては、特にローダミン、シアニン色素等が好ましく使用可能である。

【0048】本発明においては特に、ローダミン発蛍光団（Handbook of Fluorescent Probes and Research Chemicals，5th Edition MOLECULAR PROBES，INC.，1992）を導入することが好ましい。

【0049】本ローダミン発蛍光団はメタノール中において、556nmに極大吸収（モル吸光係数93．000）を有し、また576nmを極大とする蛍光を有する。

【0050】さらに本発明において好適に使用可能な発蛍光団としては、芳香族複素環式化合物または多環芳香族炭化水素がある（例えば、蛍光リン光分析、西川泰治、平木敬三著、共立出版、1989参照）。例えばアントラセン、ナフタレン、フェナントレン、キノリン、ピレン、ペリレン等またはそれらの誘導体が特に好適に使用可能である。これらの炭化水素発蛍光団には、適当な結合基を設けることにより好適にオリゴペプチドと結合可能となる。

【0051】（結合基）本発明においては、発蛍光団基とオリゴペプチドのシステインのSH基とを結合する結合基の種類においては特に制限はない。結合基を介することなく、発蛍光団を含む基が前記SH基と結合してもよいし、適当な結合基を介して結合してもよい。

【0052】この場合、発蛍光団を含む基が前記SH基と、例えば、チオエステル結合、ジチオエステル結合、チオエーテル結合（スルフィド結合）等で結合することが好ましい。本発明においては特にチオエーテル結合が好ましい。

ジアミン（例えばエチレンジアミン）との反応によりチオアミド結合（色素－$SO_2NH(CH_2)_2NH_2$）を形成し、さらにモノヨード酢酸N－オキシコハク酸イミドエステルと反応させて、得られる色素－$SO_2NH(CH_2)_2NHCOCH_2I$は容易に選択的にオリゴペプチドのシステインのSH基と反応し、チオエーテル結合を形成する。

【0055】色素－$SO_2NH(CH_2)_2NHCOCH_2$－S－システイン

本反応は温和な条件で行うことが可能であり、他のオリゴペプチド部分および蛍光色素部に変化を与えない。

【0056】さらに、オリゴペプチドに複数の反応活性点がある場合には、前記SH基にのみ反応可能とするため、反応条件、反応試薬等の選択が可能である。この選択を可能とするため、一般的な有機合成の手法が好適に使用可能である。

【0057】例えば、必要ならば、反応性の他の置換基（例えばアミノ基、フェノール基等）をあらかじめ保護しておくことは好ましい手法の1つである。

【0058】本発明においては、得られた蛍光検出キャピラリー等電点電気泳動用マーカーの純度、および、保存安定性は、等電点電気泳動法により確認可能である。

【0059】さらに必要ならば、高速液体クロマトグラフィーを用いて簡便に精製可能である。その際、逆相系カラム、特にオクタデシル基を化学結合させた充填剤を用いることで効率の良い分離精製が可能となる。検出には280nmが好適に使用可能な波長である。

【0060】（pI測定）本発明において、蛍光等電点電気泳動用マーカーのpIは、等電点電気泳動を行い、市販の等電点電気泳動用マーカーとの移動度を比較すること、あるいは標識ペプチドの泳動位置のpHを直接測定することにより測定可能である。

【0061】等電点電気泳動はポリアクリルアミドを支持体とするスラブ（平面状）ゲル等電点電気泳動が好ましく使用可能である（Righetti,P.G.,Isoelectric Focusing:Theory, Methodology and Applications, Elsevier, Amsterdam, 1983参照）。

【0062】染色方法による検出は一般の方法（例えばクーマシーブリリアントブルーR250染色）を好適に使用可能である。

が、キャピラリー等電点電気泳動装置によって、それぞれのpIに応じて泳動用担体に形成されたpHの位置に濃縮されて静止する。さらにこれらの位置は、励起光照射により発生する蛍光を検出することにより確認され得る。

【0066】本発明においては、励起光源は特には制限されないが、安定した光照射を可能とするレーザーの使用が特に好ましい。

【0067】例えば、本発明においては、発蛍光団であるローダミン色素に対する励起光としては、ヘリウムネオンレーザーが好適に使用可能である。

【0068】キャピラリーの一端に設けられた蛍光検出器により泳動用担体に形成されたpHのグラジエントを確認することができる。

【0069】上で説明したように、本発明の蛍光検出等電点電気泳動用マーカーは、オリゴペプチドの、発蛍光団を有する基を結合させることにより合成し、pIが均一でしかも高い安定性を有する。従って蛍光検出等電点泳動において、泳動用担体に形成されたpHのグラジエントに基づき、泳動された試料物質の等電点を確認する際の高感度分析用のマーカーとなる。

【0070】従って本発明の蛍光検出等電点電気泳動用マーカーは、キャピラリー等電点泳動方法にも使用可能となる。

【0071】以下、実施例に基づき本発明をさらに具体的に説明するが、本発明はその要旨を越えない限り、以下の実施例に限定されるものではない。

【0072】（蛍光検出等電点電気泳動用マーカーキット）上記説明したように、本発明に係る蛍光等電点電気泳動用マーカーに使用可能なオリゴペプチドは、適時選択し、組合せて使用可能である。

【0073】すなわち、本発明に係るマーカーは、pIとして3から10の範囲にわたり計算値と実測値の良好な一致を示すものであり（具体的には表1に挙げられている）、かつ良好な焦点性を示すものである。さらに、本発明に係るマーカーは、低分子量のオリゴペプチドであって、それらを複数組合せて種々の等電点電気泳動条件下で使用してもそれぞれ独立にそれぞれのpI値に焦点化するものである。上記組合せとしては、具体的にはマーカーとして使用したい範囲内にある本発明に係るマ

ーし．かつそのｐｉも略等間隔となっているものである。

【００７５】また、同様に．１，４，６，７，８，９，１０，１１，１２，１３，１４，１５の１２種類の組合せ（Gly-Cys-Tyr-Lys-Arg、Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys．Gly-Cys-Glu-Tyr-Tyr-Lys-Lys、Gly-Cys-Glu-His-His-His-Arg、Gly-Cys-Glu-His-Arg、Gly-Cys-Glu-His-His．Gly-Cys-Glu-Arg．Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His、Gly-Cys-Glu-Glu-His．Gly-Cys-Asp-Asp-Arg、Gly-Cys-Glu-Glu）も好ましいキットの１例である。

【００７６】

【実施例】

（蛍光検出等電点電気泳動用マーカーの合成）

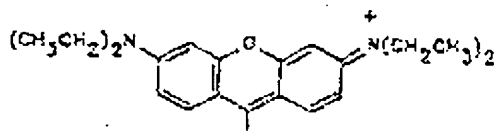
（１）Gly-Cys-Tyr-Lys-Arg、Gly-Cys-Tyr-Lys-Lys、Gly-Cys-Tyr-Tyr-Lys-Lys、Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys、Gly-Cys-Tyr-Lys、Gly-Cys-Glu-Tyr-Tyr-Lys-Lys．G*ly-Cys-Glu-His-His-His-Arg、Gly-Cys-Glu-His-Arg．Gly-Cys-Glu-His-His、Gly-Cys-Glu-Arg、Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His．Gly-Cys-Glu-Glu-His、Gly-Cys-Asp-Asp-Arg、Gly-Cys-Glu-Glu．Gly-Cys-Asp-Asp-Aspの調製。

【００７７】ペプチドは．Applied Biosystems社製ペプチド自動合成装置によりＢｏｃ法に基づいて合成し（例えば．新生化学実験講座１．タンパク質Ｖｉ、日本生化学会編、１９９２年を参照）、得られたオリゴペプチドは．高速液体クロマトグラフにより精製した。高速液体クロマトグラフに用いた条件は、ワイエムシー社製カラムＳ－５、１２０Ａ　ＯＤＳ（３０ｍｍ内径ｘ２５０ｍｍ全長、）を用い、流速は２０ｍｌ／分、検出波長は２２０ｎｍ、移動相はアセトニトリル勾配－０．１％トリフルオロ酢酸で以下のグラジエント／80分条件で用いた。

【００７８】

オリゴペプチド	HPLC移動相勾配条件
Gly-Cys-Tyr-Lys-Arg	3%～ 23%
Gly-Cys-Tyr-Lys-Lys	4.5%～24.5%
Gly-Cys-Tyr-Tyr-Lys-Lys	2.5%～22.5%
Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys	1%～ 19%
Gly-Cys-Tyr-Lys	3.7%～23.7%
Gly-Cys-Glu-Tyr-Tyr-Lys-Lys	2.3%～22.3%
Gly-Cys-Glu-His-His-His-Arg	1%～ 18%
Gly-Cys-Glu-His-Arg	0%～ 5%
Gly-Cys-Glu-His-His	0.1%～ 10%
Gly-Cys-Glu-Arg	1%～ 10%
Gly-Cys-Glu-His	0%～ 5%
Gly-Cys-Asp-Asp-His-His	1%～ 15%
Gly-Cys-Glu-Glu-His	1%～ 13%
Gly-Cys-Asp-Asp-Arg	1%～18.5%
Gly-Cys-Glu-Glu	1%～ 19%
Gly-Cys-Asp-Asp-Asp	0.5%～ 10%

【００７９】（２）リサミンローダミンＢ（ＬＲＢ）ヨードアセタミドの調製（Alexander,H., Lewis,A.S., and Ram,R.C., J.Medicinal Chem.,1976,Vol19,1279-1283）



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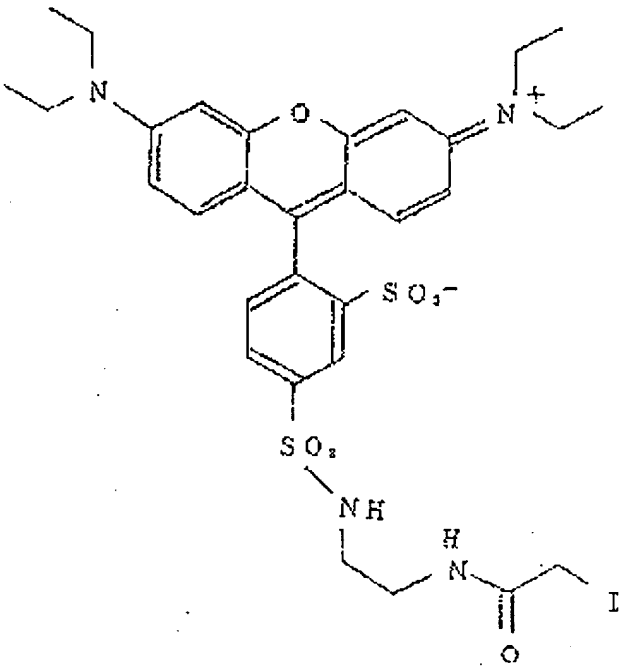
液（pH7.5）1.6mlに溶解し、570nmにおけるモル吸光係数を93,000として吸光度測定によってその濃度を決定した。ＬＲＢアミン粗製品150nmolを25%アセトニトリル－0.1%トリフルオロ酢酸溶液で平衡化した逆相クロマトグラフカラム（東ソーODS-80Ts、直径4.6mm、長さ25cm）にかけ、30分間にわたって25－55%のアセトニトリルの濃度直線勾配をかけて溶出し、280nmの吸収をモニターすることで検出を行った。最も大きなピークを分取し（127nmol）、精製ＬＲＢアミンとして以下の操作に用いた。

【００８２】（ｂ）ＬＲＢアミンのヨードアセチル化（ａ）で調製した画分を減圧下に乾固し、その後、アセトニトリルと0.1Mリン酸ナトリウム緩衝液(pH7.5)の1:4混合液88μｌに溶解し、これに10mMモノヨード酢酸Ｎ－オキシコハク酸イミドエステルのジメチルホルムアミド溶液12μｌを添加し、室温にて１時間反応させた。反応液の全量を（ａ）で説明した同じ条件にて逆相クロマトグラフにかけ、溶出した主ピークをＬＲＢ－ヨードアセタミドとして分取し（70nmol）、ペプチドの標識に用いた。以下にＬＲＢ－ヨードアセタミドの構造を示した。

【００８３】

【化２】



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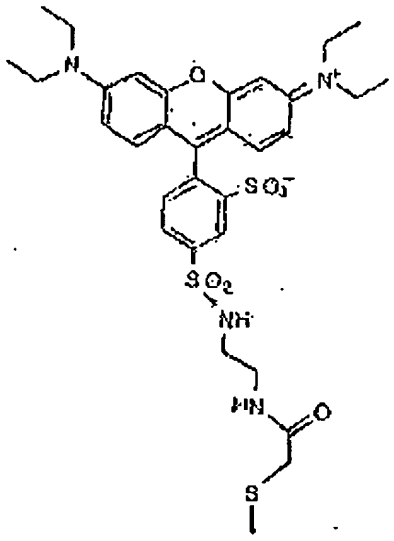
【００８４】（３）ペプチドの標識

（２）で調製した精製色素（ＬＲＢ－ヨードアセタミド）25nmolを含むカラム溶液を試験管中で乾固し、その後、アセトニトリル20μｌに溶解した。これに、（１）で調製したペプチド水溶液１０μｌ（濃度5mM、50nmol）と緩衝液（0.1M Na-Pi、5mM EDTA、pH7.5）を加え、暗所にて一晩室温で反応させた。反応液を、25%アセトニトリル－0.1%トリフルオロ酢酸溶液で平衡化した逆相クロマトグラフカラム（東ソー ODS-80Ts、直径4.6mm、長さ25cm）にかけ、1ml／分の流速にて、30分間にわたって25－55%のアセトニトリルの濃度直線勾配をかけて標識ペプチドを溶出した。分取した。検出は280nmの吸収にて行った。標識ペプチドの収量は、80%であった。標識ペプチドの濃度は、570nmにおけるモル吸光係数を93,000として、吸光度測定によって決定した。以下に標識されたオリゴペプチドGly-Cys-Glu-Tyr-Tyr-Lys-Lysの構造を示す。

【００８５】

【化３】



H－Gly－Cys－Glu－Tyr－Tyr－Lys－Lys－OH

【００８６】（蛍光物質標識ペプチドのｐｉ）Gly-Cys-Tyr-Lys、Gly-Cys-Glu-Tyr-Tyr-Lys-Lys、Gly-Cys-Glu-His-His-His-Arg、Gly-Cys-Glu-His-Arg、Gly-Cys-Glu-His-His、Gly-Cys-Glu-Arg、Gly-Cys-Glu-His、Gly-Cys-Asp-Asp-His-His、Gly-Cys-Glu-Glu-His、Gly-Cys-Asp-Asp-Arg、Gly-Cys-Glu-Glu、Gly-Cys-Asp-Asp-Aspの上記蛍光物質標識オリゴペプチドは、Ampholine PAG Plate(pH3.5-9.5、ファルマシアバイオテク社製）（5%T、3%C、24.5mm×110mm大きさ、1mm厚さ）を用いて等電点電気泳動を行い、市販の等電点電気泳動用マーカー（ｐＩカリブレーションキット３－１０、ファルマシアバイオテク社製）との移動度を比較した。

【００８７】泳動条件は３００Ｖの電圧にて電気泳動を開始した後、３０分後に１．５００Ｖへ電圧を増加し、５０分間泳動を行った。

【００８８】泳動終了後、蛍光物質標識オリゴペプチドについては、オレンジ色のフィルターを通して紫外線を照射することにより写真撮影を行い、泳動位置を確認した。

【００８９】その後、市販の等電点電気泳動用マーカーの泳動位置を知るためにゲルを、ページブルー８３染色液（ＣＢＢ－Ｒ２５０、第一化学薬品社製）にて染色を行った。

【００９０】染色後、メタノールと酢酸の水溶液にて＊いては以下の方法でｐｉを測定し、実測知を決定した。

【００９２】すなわち、１x６５x１２５mmの合成シリカ板上に、ｐＨ勾配形成のための両性担体としてファルマシアバイオテク社製のPharmalyte8-10.5を終濃度として原液の１／１６希釈で含む１x５０x１１５mmのポリアクリルアミドゲル（4.2%T、4.8%C）を作製した。ゲルの乗ったシリカ板は恒温水を循環させた水平な冷却板上に置き、空気中の炭酸ガスの影響を避けるため、窒素ガスを満たしたグローブボックス中で電気泳動を行った。短片に沿って電極を置き、試料としてそれぞれのペプチド約0.5 nmolを陰極側および陽極側から１cmの位置のゲル表面に添加し、最終的に添加した試料が１箇所に焦点化するまで約１００V/cmの電圧で焦点化した（約２時間）。焦点化が完了した後、電圧をかけたままの状態でゲルの温度が２５℃になるようにゲル冷却板に循環する水の温度を調整し、ｐＨ勾配に沿って１cm間隔にゲル表面のｐＨを測定した。ゲルの温度とｐＨの測定には東亜電波工業株式会社製ＭＥＴＯＸＹｐＨ計（HM-17MX型）を用いた。ゲルの位置に対してｐＨをプロットしてゲル中のｐＨ勾配を求め、蛍光物質標識オリゴペプチドの焦点化位置からそれぞれの蛍光標識オリゴペプチドの等電点を決定した。

【００９３】得られた蛍光物質標識オリゴペプチドと市販の等電点電気泳動用マーカーの泳動像を図１に示し

1 Gly-Cys-Tyr-Lys-Arg	10.02	9.00
2 Gly-Cys-Tyr-Lys-Lys	9.76	8.98
3 Gly-Cys-Tyr-Tyr-Lys-Lys	9.52	8.91
4 Gly-Cys-Tyr-Tyr-Tyr-Lys-Lys	9.36	8.85
5 Gly-Cys-Tyr-Lys	8.62	8.30
6 Gly-Cys-Glu-Tyr-Tyr-Lys-Lys	8.46	8.36
7 Gly-Cys-Glu-His-His-His-Arg	7.34	7.67
8 Gly-Cys-Glu-His-Arg	7.04	7.24
9 Gly-Cys-Glu-His-His	6.42	6.71
10 Gly-Cys-Glu-Arg	6.06	6.04
11 Gly-Cys-Glu-His	5.48	5.60
12 Gly-Cys-Asp-Asp-His-His	5.24	5.34
13 Gly-Cys-Glu-Glu-His	4.54	4.35
14 Gly-Cys-Asp-Asp-Arg	4.16	4.10
15 Gly-Cys-Glu-Glu	3.82	3.59
16 Gly-Cys-Asp-Asp-Asp	3.38	<3.50(*)

(*)蛍光標識オリゴペプチドの泳動位置が、用いた市販の等電点電気泳動用マーカーの移動度の範囲（ｐＩ9.30～3.50）を越えていることから、実測値を得ることができなかった。

【００９６】（蛍光検出キャピラリー等電点電気泳動）得られた蛍光物質標識オリゴペプチドを蛍光検出キャピラリー等電点電気泳動によって分離・検出を行った（Ｈｊｅｒｔｅｎ，Ｓ．，Ｊｏｕｒｎａｌ ｏｆ Ｃｈｒｏｍａｔｏｇｒａｐｈｙ，３４７，１９１－１９８，１９８５）。内面をポリアクリルアミドでコーティング処理を施した内径0.05ｍｍ、外径0.375ｍｍ、全長22ｃｍの溶解シリカキャピラリー（ジーエルサイエンス社製）を、２５倍希釈Pharmalyte（pH3-10、ファルマシアバイオテク社製）で満たし、陽極から上記得られたオリゴペプチド（Gly-Cys-Glu-Tyr-Tyr-Lys-Lys、1×10^{-4}M）を含む２５倍希釈Pharmalyte（pH3-10）溶液を落差5cmの静水圧で１分間流すことにより導入した。

【００９７】陽極液として20mMリン酸、陰極液として20mMのNaOHを用い、両電極槽を同じ高さにして５分間、５００Ｖ／ｃｍで焦点化した。焦点化したマーカーは５００Ｖ／ｃｍの電場を加えつつ陽極槽を陰極槽に対して５ｃｍ高くすることにより、陰極側へ移動させ、陰極端から２ｃｍの位置でレーザー励起による蛍光検出を行った。

【０１００】以上の結果によれば、本発明により、オリゴペプチドに発蛍光団色素を含むことにより、蛍光物質標識オリゴペプチドが得られる。この際、オリゴペプチドと発蛍光団は温和な条件下で結合され得るため、オリゴペプチドおよび蛍光物質に対して障害を与えることなく、さらにオリゴペプチドに結合させる蛍光物質の数と位置も特定でき、得られる発蛍光団色素を含むオリゴペプチドは、安定性の高い、均一な単一のｐＩを有することとなる。従って、本発明において得られる蛍光検出キャピラリー等電点電気泳動用マーカーは、試料物質の等電点を推定する際のマーカーとして使用可能となる。

【０１０１】

【発明の効果】本発明に係る、蛍光検出等電点電気泳動法において用いる蛍光検出等電点電気泳動用マーカーは、ＳＨ基に発蛍光団色素を結合したシステインを少なくとも１つ含むオリゴペプチドであることを特徴とする。従って、得られた本発明に係るマーカーは通常の高分子蛋白に基づくマーカーに比較してその化学的安定性において優れたものとなる。さらにＳＨ基に発蛍光団色素を結合したシステインを少なくとも１つ含むものであ

配列番号：1
配列の長さ：5
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Tyr Lys Arg
5

配列番号：2
配列の長さ：5
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Tyr Lys Lys
5

配列番号：3
配列の長さ：6
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Tyr Tyr Lys Lys
5

配列番号：4
配列の長さ：7
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Tyr Tyr Tyr Lys Lys
5

配列番号：5
配列の長さ：4
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Tyr Lys
配列番号：6

トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Glu His His His Arg
5

配列番号：8
配列の長さ：5
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Glu His Arg
5

配列番号：9
配列の長さ：5
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Glu His His
5

配列番号：10
配列の長さ：4
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Glu Arg
配列番号：11
配列の長さ：4
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Glu His
配列番号：12
配列の長さ：6
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド

配列
Gly Cys Glu Glu His
5

配列番号：14
配列の長さ：5
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Asp Asp Arg
5

配列番号：15
配列の長さ：4
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly-Cys-Glu-Glu
配列番号：16

＊配列の長さ：5
配列の型：アミノ酸
トポロジー：直鎖状
配列の種類：ペプチド
配列
Gly Cys Asp Asp Asp
5

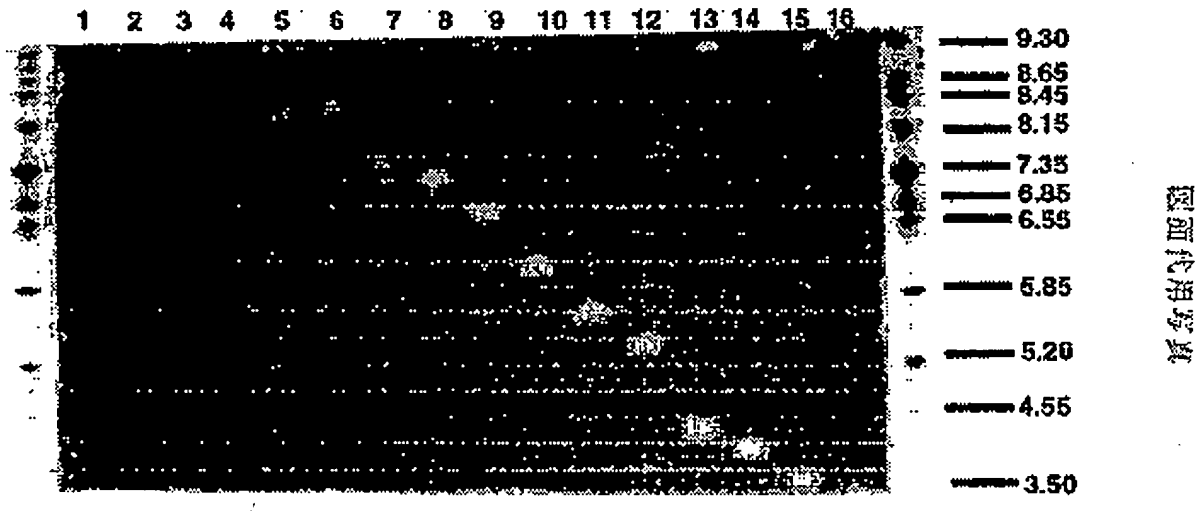
【図面の簡単な説明】

【図1】本発明に係る16種類の蛍光物質標識ペプチドと市販の等電点電気泳動用マーカーの電点電気泳動を行った際の電気泳動像を示す電気泳動写真である。図中の番号1～16は、表1中に番号1～16で示されるオリゴペプチドに対応する。

【図2】本発明に係る蛍光物質標識ペプチドの、蛍光検出キャピラリー等電点電気泳動を行った際の移動時間と蛍光強度を示す図である。

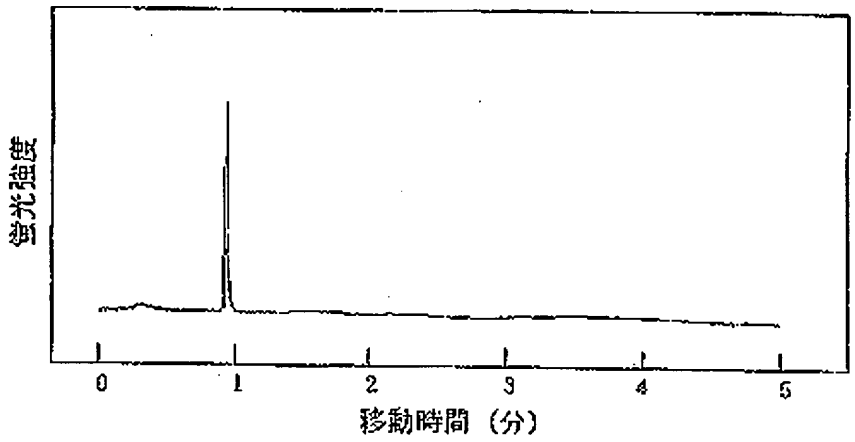
＊

【図1】



図面代用写真

【図2】



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